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Output ที่ได้จากโครงการ

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ภาคผนวก

Particle size and zeta-potential of the pDNA/CS derivative complexes

Table 1 Particle size and zeta-potential of the pDNA/TM_{57}-Bz_{43}-CS complexes

N/P ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD	Zeta deviation	AVG	SD
0.7	1530	1460	69.0	0.483	0.528	0.048	-31.0	-29.0	4.49	22.6	21.1	4.91
	1450			0.579			-23.9			21.9		
	1390			0.521			-32.1			30.7		
1.5	1080	1070	34.1	0.400	0.467	0.060	-35.5	-35.7	3.29	4.13	6.65	3.04
	1090			0.513			-39.2			5.78		
	1030			0.489			-32.6			10.0		
3	2730	2840	356	0.680	0.706	0.023	-1.92	-4.01	1.82	6.78	10.3	5.80
	2560			0.719			-4.87			7.07		
	3240			0.720			-5.24			17.0		
6	319	329	7.94	0.388	0.376	0.033	28.2	30.3	2.05	16.2	10.9	4.67
	333			0.400			32.3			8.79		
	334			0.339			30.3			7.58		
8	393	388	14.0	0.301	0.327	0.023	25.9	26.2	0.526	23.5	18.2	6.95
	399			0.341			26.8			10.3		
	372			0.339			26.0			20.8		
11	292	293	10.7	0.204	0.202	0.006	30.4	32.4	3.14	5.67	9.39	4.63
	283			0.195			36.0			7.93		
	304			0.206			30.7			14.6		
17	346	351	5.70	0.287	0.317	0.032	35.5	34.2	1.11	134	76.8	62.8
	357			0.313			33.7			87.6		
	351			0.350			33.5			9.24		
23	361	364	2.99	0.362	0.346	0.030	26.7	30.8	4.03	19.1	12.8	6.17
	367			0.312			34.7			12.4		
	365			0.364			30.9			6.81		
34	269	278	7.98	0.263	0.237	0.045	29.5	29.7	2.71	120	65.1	57.1
	284			0.185			27.1			69.1		
	282			0.262			32.5			6.19		
45	418	424	5.87	0.375	0.364	0.038	39.2	37.8	1.45	13.2	12.2	2.61
	424			0.322			36.3			9.18		
	430			0.396			37.7			14.1		

Table 2 Particle size and zeta-potential of the pDNA/TM_{47}-Bz_{43}-CS complexes

N/P ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD	Zeta deviation	AVG	SD
0.7	510	486	21.3	0.426	0.464	0.072	-18.8	-27.2	7.65	19.2	14.7	6.07
	477			0.547			-33.9			7.80		
	470			0.419			-28.9			17.2		
1.5	3770	3143	542.8	0.758	0.722	0.149	6.11	12.9	7.43	6.63	4.52	1.84
	2820			0.850			11.7			3.71		
	2840			0.558			20.8			3.24		
3	390	411	18.7	0.317	0.322	0.005	25.7	27.5	3.5	14.9	9.81	4.77
	420			0.322			31.6			5.53		
	424			0.327			25.3			8.96		
6	544	552	18.7	0.363	0.375	0.025	38.9	32.0	5.96	54.2	26.0	24.4
	574			0.404			28.0			11.5		
	539			0.357			29.2			12.3		
9	461	456	7.66	0.452	0.423	0.048	33.0	34.6	1.43	111	42.2	59.9
	461			0.449			35.6			5.75		
	448			0.369			35.2			9.41		
11	501	514	14.8	0.346	0.367	0.036	31.2	33.6	2.10	26.0	19.9	5.38
	511			0.409			34.1			18.0		
	530			0.345			35.3			15.8		
17	257	253	6.20	0.343	0.381	0.068	30.7	30.6	0.52	35.8	52.8	43.8
	256			0.460			30.0			20.1		
	246			0.340			31.1			103		
23	260	289	25.8	0.407	0.389	0.030	35.2	34.4	0.79	16.0	22.5	14.2
	295			0.355			33.6			12.8		
	311			0.406			34.2			38.8		
34	260	257	2.47	0.347	0.308	0.042	34.8	34.6	0.72	23.2	25.3	4.33
	256			0.264			33.8			30.3		
	256			0.314			35.2			22.4		
46	377	345	29.1	0.722	0.455	0.233	37.8	36.9	0.96	74.7	40.3	32.4
	320			0.343			35.8			35.8		
	337			0.299			37.0			10.3		

Table 3 Particle size and zeta-potential of the pDNA/TM_{57}-Bz_{18}-CS complexes

N/P ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD	Zeta deviation	AVG	SD
0.8	648	651	15.3	0.471	0.471	0.033	-43.0	-36.2	5.92	13.2	17.2	3.76
	667			0.438			-32.9			20.6		
	637			0.505			-32.6			18.0		
1.5	950	850	100	0.493	0.433	0.058	-15.20	-10.2	5	12.7	15.7	3.22
	850			0.428			-10.20			15.3		
	750			0.377			-5.20			19.1		
3	1050	991	84.5	0.556	0.580	0.089	30.0	29.3	0.792	22.4	16.3	5.96
	1030			0.679			28.4			15.9		
	894			0.506			29.3			10.5		
6	749	748	0.507	0.410	0.350	0.079	34.1	30.9	4.10	11.0	12.1	3.68
	748			0.390			26.2			16.2		
	748			0.261			32.2			9.03		
9	361	363	3.56	0.238	0.288	0.049	21.9	21.3	0.951	8.02	7.89	1.37
	362			0.337			20.2			9.19		
	367			0.289			21.8			6.45		
12	546	570	21.5	0.336	0.344	0.027	35.9	33.8	1.83	10.5	25.4	19.0
	582			0.322			32.5			46.7		
	584			0.374			33.0			19.0		
19	461	452	8.29	0.126	0.188	0.055	31.3	31.3	1.35	92.1	73.0	40.8
	444			0.214			32.7			26.2		
	450			0.225			30.0			101		
25	605	609	14.5	0.371	0.383	0.081	34.0	33.8	0.609	8.88	11.5	2.57
	597			0.309			33.2			14.0		
	625			0.470			34.4			11.7		
37	350	353	3.93	0.286	0.271	0.013	22.8	23.5	0.611	23.4	22.5	0.84
	352			0.261			23.8			22.2		
	358			0.268			24.0			21.8		
49	647	559	78.1	0.533	0.504	0.027	35.8	31.7	3.53	58.5	48.4	14.4
	499			0.480			30.1			54.9		
	531			0.497			29.3			31.9		

Table 4 Particle size and zeta-potential of the pDNA/TM_{43}-CS complexes

N/P ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD	Zeta deviation	AVG	SD
0.8	192	199	9.31	0.310	0.324	0.024	-8.30	-12.6	3.93	9	12.6	4.53
	209			0.352			-13.6			11.2		
	196			0.311			-16.0			17.7		
1.5	293	252	74.6	0.476	0.409	0.081	-10.9	-12.2	2.39	13.0	13.4	6.80
	310			0.495			-15			6.77		
	314			0.426			-10.7			20.4		
3	525	539	12.6	0.295	0.341	0.041	31.2	27.9	3.25	4.66	12.7	6.68
	547			0.371			27.7			17.9		
	546			0.357			24.7			12.6		
7	278	267	10.4	0.536	0.458	0.067	24.8	29.3	4.59	21.4	17.0	4.6
	258			0.420			29.2			17.5		
	266			0.418			34.0			12.2		
10	198	193	4.61	0.343	0.339	0.030	29.0	29.9	0.970	15.7	16.5	2.73
	193			0.307			30.9			19.6		
	189			0.366			29.7			14.3		
13	220	217	2.86	0.312	0.321	0.012	29.7	24.2	7.79	18.6	24.5	8.82
	215			0.334			15.3			20.2		
	215			0.316			27.5			34.6		
20	250	253	2.40	0.319	0.304	0.042	26.5	26.53	1.25	110	67.9	47.7
	255			0.256			25.3			77.6		
	254			0.337			27.8			16.4		
27	160	176	4.3	0.335	0.333	0.016	28.3	28.3	0.8	82.3	82.5	32.9
	182			0.315			27.5			115		
	186			0.348			29.1			49.7		
40	287	291	4.68	0.343	0.365	0.019	25.7	33.4	6.81	63.3	47.4	18.8
	297			0.376			35.8			52.2		
	290			0.377			38.7			26.7		
54	327	319	7.1	0.362	0.389	0.057	25.2	32.7	8.0	35.0	36.2	4.24
	314			0.350			41.2			40.9		
	316			0.455			31.7			32.7		

Table 5 Particle size and zeta-potential of the pDNA/CS complexes

N/P ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD	Zeta deviation	AVG	SD
1	628	586	36.9	0.534	0.657	0.126	-22.7	-23.0	0.966	9.78	8.76	1.47
	572			0.774			-24.1			7.08		
	558			0.717			-22.2			9.43		
2	821	758	60.4	0.555	0.666	0.106	-23.5	-23.1	2.72	136	95.7	40.2
	701			0.677			-20.2			55.1		
	750			0.766			-25.6			96.6		
4	508	488	19.5	0.470	0.424	0.041	54.9	50.7	4.73	16.2	17.4	4.2
	470			0.392			51.5			13.9		
	486			0.411			45.6			22.1		
8	591	579	16.9	0.486	0.465	0.023	50.6	51.7	1.13	24.4	29.5	4.46
	586			0.441			52.9			32.6		
	560			0.468			51.6			31.5		
12	572	581	19.1	0.432	0.433	0.019	54.1	53.9	0.184	28.8	20.6	7.18
	568			0.452			54.0			17.5		
	603			0.414			53.7			15.5		
16	624	687	74.5	0.468	0.492	0.028	46.8	54.5	7.04	38.5	32.2	5.48
	668			0.522			60.7			28.7		
	769			0.485			56.1			29.3		
24	565	557	46.8	0.529	0.477	0.058	55.8	50.0	6.57	15.2	42.2	23.6
	507			0.414			42.9			58.7		
	600			0.486			51.4			52.8		
32	526	531	7.03	0.465	0.447	0.015	55.6	52.2	3.08	27.2	25.7	5.41
	530			0.439			51.3			30.2		
	539			0.438			49.6			19.7		
48	398	437	49.3	0.372	0.398	0.023	53.6	55.8	2.09	62.1	69.1	7.84
	421			0.416			57.8			77.6		
	492			0.407			56.0			67.7		
64	626	660	38.8	0.681	0.581	0.126	49.6	52.0	3.14	15.6	37.2	30.1
	652			0.440			51.0			55.6		
	703			0.623			55.6			40.3		

Table 6 Particle size and zeta-potential of the pDNA/PEI complexes

PEI cpx.	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD	Zeta deviation	AVG	SD
1:1	270	271	1.87	0.315	0.318	0.032	35.9	36.4	1.05	14.9	16.4	1.38
	270			0.288			35.7			17.6		
	273			0.352			37.6			16.8		

In vitro transfection efficiency

1. Preliminary study

Table 7 Effect of post-transfection time of TM_{57}-Bz_{43}-CS

Sample	Post-transfection time (h)								
	24 h			48 h			72 h		
	cell/cm^2	AVG	SD	cell/cm^2	AVG	SD	cell/cm^2	AVG	SD
PEI	35.263	31.930	2.993	187.895	180.175	14.292	630.526	762.632	115.455
	29.474			188.947			813.158		
	31.053			163.684			844.211		
3	0.000	0.000	0.000	9.474	11.754	1.993	39.474	38.070	2.431
	0.000			12.632			35.263		
	0.000			13.158			39.474		
6	0.000	0.000	0.000	42.105	32.105	8.664	272.105	190.000	75.460
	0.000			27.368			174.211		
	0.000			26.842			123.684		
11	0.000	0.000	0.000	83.158	96.491	23.094	238.421	283.333	78.247
	0.000			123.158			373.684		
	0.000			83.158			237.895		
23	0.000	0.000	0.000	151.579	134.035	16.079	668.947	532.632	122.002
	0.000			120.000			495.263		
	0.000			130.526			433.684		
45	0.000	0.000	0.000	0.000	0.000	0.000	143.684	123.509	33.587
	0.000			0.000			84.737		
	0.000			0.000			142.105		

Table 8 Effect of post-transfection time of TM_{47}-Bz_{43}-CS

Sample	Post-transfection time (h)								
	24 h			48 h			72 h		
	cell/cm^2	AVG	SD	cell/cm^2	AVG	SD	cell/cm^2	AVG	SD
PEI	68.947	60.175	8.227	206.842	242.807	61.384	873.684	783.860	199.962
	58.947			207.895			554.737		
	52.632			313.684			923.158		
3	0.000	0.000	0.000	3.158	2.281	1.096	17.895	14.211	3.974
	0.000			2.632			10.000		
	0.000			1.053			14.737		
6	0.000	0.000	0.000	4.211	5.614	1.993	85.263	96.316	9.861
	0.000			4.737			99.474		
	0.000			7.895			104.211		
11	0.000	0.000	0.000	115.789	108.070	9.197	391.579	435.263	40.321
	0.000			97.895			443.158		
	0.000			110.526			471.053		
23	0.000	0.000	0.000	12.632	10.175	2.191	48.947	38.947	8.664
	0.000			9.474			34.211		
	0.000			8.421			33.684		
46	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	0.000			0.000			0.000		
	0.000			0.000			0.000		

Table 9 Effect of post-transfection time of TM_{57}-Bz_{18}-CS

Sample	Post-transfection time (h)								
	24 h			48 h			72 h		
	cell/cm^2	AVG	SD	cell/cm^2	AVG	SD	cell/cm^2	AVG	SD
PEI	78.947	66.667	10.956	190.526	190.526	8.947	824.211	727.368	95.553
	63.158			181.579			633.158		
	57.895			199.474			724.737		
3	0.000	0.000	0.000	10.000	10.000	0.526	17.895	18.246	1.608
	0.000			10.526			20.000		
	0.000			9.474			16.842		
6	0.000	0.000	0.000	8.421	8.070	1.608	16.316	17.018	0.804
	0.000			6.316			16.842		
	0.000			9.474			17.895		
12	0.000	0.000	0.000	10.526	11.579	1.393	23.158	30.526	7.120
	0.000			13.158			37.368		
	0.000			11.053			31.053		
25	0.000	0.000	0.000	12.632	10.175	2.191	27.895	25.263	2.930
	0.000			9.474			25.789		
	0.000			8.421			22.105		
49	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	0.000			0.000			0.000		
	0.000			0.000			0.000		

2. *In vitro* transfection study

2.1 Effect of N/P ratio

Table 10 Transfection efficiency of TM_{57}-Bz_{43}-CS in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	1566	824.211	1203	633.158	1377	724.737	727.368	95.553
3	75	39.474	67	35.263	75	39.474	38.070	2.431
6	517	272.105	331	174.210	235	123.684	190.000	75.460
8	540	284.211	333	175.263	357	187.895	215.790	59.590
11	453	238.421	710	373.684	452	237.895	283.333	78.247
17	793	417.368	652	343.158	530	278.947	346.491	69.271
23	1083	570.000	1217	640.526	1005	528.947	579.825	56.435
34	811	426.842	657	345.789	700	368.421	380.351	41.823
45	210	110.526	180	94.737	150	78.947	94.737	15.789

Table 11 Transfection efficiency of TM_{47}-Bz_{43}-CS in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	1322	695.789	1428	751.579	1477	777.368	741.579	41.699
3	44	23.158	40	21.053	27	14.211	19.474	4.678
6	244	128.421	235	123.684	292	153.684	135.263	16.128
9	797	419.474	821	432.105	697	366.842	406.140	34.614
11	744	391.579	842	443.158	895	471.053	435.263	40.321
17	351	184.737	308	162.105	324	170.526	172.456	11.439
23	93	48.947	65	34.211	64	33.684	38.947	8.664
34	0	0.000	0	0.000	0	0.000	0.000	0.000
46	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 12 Transfection efficiency of TM_{57}-Bz_{18}-CS in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
PEI	1088	572.632	1540	810.526	1422	748.421	710.526	123.392
3	34	17.895	38	20.000	32	16.842	18.246	1.608
6	31	16.316	32	16.842	34	17.895	17.018	0.804
9	33	17.368	46	24.211	52	27.368	22.982	5.112
12	44	23.158	71	37.368	59	31.053	30.526	7.120
19	89	46.842	103	54.211	75	39.474	46.842	7.368
25	53	27.895	49	25.789	42	22.105	25.263	2.930
37	0	0.000	0	0.000	0	0.000	0.000	0.000
49	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 13 Transfection efficiency of TM_{43}-CS in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
PEI	1782	937.895	1653	870.000	1800	947.368	918.421	42.201
3	2	1.053	4	2.105	2	1.053	1.404	0.608
7	12	6.316	13	6.842	11	5.789	6.316	0.526
10	73	38.421	63	33.158	68	35.789	35.789	2.632
13	128	67.368	145	76.316	130	68.421	70.702	4.890
20	100	52.632	98	51.579	82	43.158	49.123	5.193
27	57	30.000	60	31.579	48	25.263	28.947	3.287
40	11	5.789	14	7.368	15	7.895	7.018	1.096
54	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 14 Transfection efficiency of CS in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
PEI	1573	827.895	1728	909.474	1623	854.211	863.860	41.637
4	77	40.526	103	54.211	109	57.368	50.702	8.953
8	10	5.263	15	7.895	12	6.316	6.491	1.325
12	8	4.210	6	3.158	10	5.263	4.211	1.053
16	9	4.737	5	2.632	4	2.105	3.158	1.393
24	0	0.000	0	0.000	0	0.000	0.000	0.000
32	0	0.000	0	0.000	0	0.000	0.000	0.000
48	0	0.000	0	0.000	0	0.000	0.000	0.000
64	0	0.000	0	0.000	0	0.000	0.000	0.000

2.2 Effect of pH

Table 15 Transfection efficiency of TM_{57}-Bz_{43}-CS in medium without serum, pH 6.5

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23	1280	673.684	1268	667.368	1040	547.368	629.474	71.175

Table 16 Transfection efficiency of CS in medium without serum, pH 6.5.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
4	732	385.263	582	306.316	615	323.684	345.789	55.824

2.3 Effect of serum

Table 17 Transfection efficiency of TM_{57}-Bz_{43}-CS in medium with serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2	(cell/cm^2)	
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
PEI	113	59.474	207	108.947	150	78.947	82.456	24.923
8	271	142.632	132	69.474	111	58.421	90.175	45.763
11	106	55.789	119	62.632	120	63.158	60.526	4.111
17	87	45.789	59	31.053	60	31.579	36.140	8.361
23	62	32.632	70	36.842	72	37.895	35.789	2.785
34	53	27.895	60	31.579	47	24.737	28.070	3.424
45	68	35.789	52	27.368	40	21.053	28.070	7.393

Cytotoxicity of CS derivatives and pDNA/CS derivatives complexes

1. Cytotoxicity of CS derivatives on Huh7 cells

Table 18 Cytotoxicity of TM_{57}-Bz_{43}-CS on Huh7 cells

n	Concentration of TM_{57}-Bz_{43}-CS (mg/ml)			
	0.001	0.01	0.1	1
1	85.068	70.135	33.871	8.912
2	100.142	83.290	29.533	9.623
3	95.662	74.828	28.680	10.334
4	99.368	74.107	35.017	11.617
5	94.316	75.303	37.810	10.155
6	95.380	72.245	36.613	8.825
7	93.784	70.650	35.150	8.692
8	94.050	67.592	31.694	9.756
AVG	94.721	73.519	33.546	9.739
SD	4.582	4.7381	3.287	0.977

Table 19 Cytotoxicity of TM_{47}-Bz_{43}-CS on Huh7 cells

n	Concentration of TM_{47}-Bz_{43}-CS (mg/ml)			
	0.001	0.01	0.1	1
1	88.525	75.647	7.553	8.101
2	89.347	84.278	9.334	12.896
3	100.308	80.716	10.978	12.759
4	76.743	80.853	17.006	10.978
5	82.771	77.291	8.512	7.827
6	86.205	81.939	20.929	6.423
7	84.996	87.201	19.365	8.557
8	89.405	89.903	19.791	6.281
AVG	87.288	82.228	14.183	9.228
SD	6.738	4.778	5.628	2.652

Table 20 Cytotoxicity of TM_{57}-Bz_{18}-CS on Huh7 cells

n	Concentration of TM_{47}-Bz_{43}-CS (mg/ml)			
	0.001	0.01	0.1	1
1	120.587	108.315	29.306	14.236
2	93.030	110.684	25.215	15.743
3	115.850	113.267	34.688	13.805
4	98.843	126.399	28.660	15.743
5	110.038	110.038	22.632	17.896
6	91.182	91.965	57.691	10.476
7	93.743	89.547	51.718	13.321
8	93.885	96.089	57.265	9.552
AVG	102.145	105.788	38.397	13.846
SD	11.608	12.410	14.732	2.773

Table 21 Cytotoxicity of TM_{43}-CS on Huh7 cells

n	Concentration of TM_{43}-CS (mg/ml)			
	0.001	0.01	0.1	1
1	97.473	116.400	37.250	4.234
2	117.798	93.494	31.980	5.310
3	101.237	77.363	38.755	1.869
4	82.955	88.869	43.379	0.578
5	94.569	91.235	37.895	1.869
6	95.645	90.805	51.445	10.579
7	126.616	100.376	38.325	3.374
8	99.301	91.020	50.800	6.923
AVG	101.949	93.695	41.229	4.342
SD	13.847	11.160	6.841	3.248

Table 22 Cytotoxicity of CS on Huh7 cells

n	Concentration of CS (mg/ml)			
	0.001	0.01	0.1	1
1	88.654	76.072	83.385	76.502
2	89.300	90.483	88.117	67.254
3	84.460	75.642	94.139	67.361
4	100.591	91.773	96.720	73.599
5	93.494	84.030	84.138	68.114
6	113.604	85.858	91.020	68.114
7	105.646	108.657	92.203	69.835
8	103.818	96.075	84.030	80.481
AVG	97.446	88.574	89.219	71.407
SD	10.048	10.856	5.080	4.941

2. Cytotoxicity of pDNA/CS derivatives complexes

Table 23 Cytotoxicity of pDNA/TM_{57}-Bz_{43}-CS complexes on Huh7 cells

n	N/P ratio								
	control	3	6	8	11	17	23	34	45
1	90.042	97.460	97.138	88.430	83.108	71.498	71.336	43.761	22.637
2	108.587	91.010	103.427	96.009	96.170	85.366	73.433	49.567	30.216
3	91.494	95.203	100.685	101.330	76.819	67.305	68.273	41.665	21.508
4	96.493	95.203	100.524	99.718	82.624	78.432	63.274	40.859	29.893
5	114.392	95.041	97.621	97.621	78.432	69.563	66.660	43.439	21.185
6	100.847	100.685	101.008	102.137	89.881	73.433	60.371	39.569	21.508
7	99.557	102.782	102.137	94.396	89.236	67.789	59.887	35.053	22.314
8	98.589	102.620	98.428	81.012	91.171	79.561	71.175	43.278	36.827
AVG	100	97.501	100.121	95.082	85.930	74.118	66.800	42.149	25.761
SD	8.173	4.192	2.213	7.174	6.729	6.429	5.175	4.131	5.833

Table 24 Cytotoxicity of pDNA/TM_{47}-Bz_{43}-CS complexes on Huh7

n	N/P ratio								
	control	3	6	9	12	19	25	37	49
1	102.788	92.220	90.544	80.705	75.749	69.700	65.182	68.461	57.311
2	99.727	98.779	91.856	80.924	76.114	72.105	67.660	67.441	53.521
3	94.261	87.774	90.107	75.531	72.761	64.744	60.445	53.011	53.011
4	102.278	96.593	73.271	76.842	78.592	71.012	66.566	56.363	49.294
5	104.245	95.281	91.054	76.988	76.114	61.246	60.007	61.611	53.594

6	107.015	93.969	86.608	76.259	80.122	63.433	66.931	63.651	47.035
7	94.115	105.849	97.759	63.797	77.571	73.563	70.283	56.655	49.367
8	95.573	80.997	95.791	66.639	84.276	74.073	75.166	64.963	52.063
AVG	100	93.933	89.624	74.711	77.662	68.735	66.530	61.520	51.899
SD	4.891	7.393	7.453	6.229	3.446	4.919	4.950	5.636	3.227

Table 25 Cytotoxicity of pDNA/TM_{57}-Bz_{18}-CS complexes on Huh7 cells

n	N/P ratio								
	control	3	6	8	11	17	23	34	45
1	102.788	92.220	84.349	90.544	96.175	86.171	89.988	73.075	67.810
2	99.727	106.432	92.657	76.551	96.043	80.183	66.955	65.046	62.874
3	94.261	90.398	84.932	79.393	81.367	69.587	74.128	60.966	66.165
4	102.278	96.520	88.503	79.612	81.301	73.931	82.552	63.335	63.598
5	104.245	101.257	84.203	81.070	70.508	72.680	65.309	64.980	62.085
6	107.015	96.957	89.669	86.608	84.921	65.046	69.126	67.613	63.861
7	94.115	93.022	87.702	82.527	85.645	63.138	57.280	65.046	66.362
8	95.573	95.281	63.214	72.761	66.626	83.276	71.364	71.496	80.972
AVG	100	96.511	84.404	81.133	82.823	74.251	72.088	66.445	66.716
SD	4.891	5.218	9.046	5.567	10.582	8.378	10.247	4.081	6.082

Table 26 Cytotoxicity of pDNA/TM_{43}-CS complexes on Huh7 cells

n	N/P ratio								
	control	4	8	12	16	24	32	48	64
1	98.621	96.468	102.568	101.581	81.666	77.181	76.463	75.477	75.387
2	89.650	97.634	86.510	100.415	77.450	79.693	78.616	76.732	59.688
3	106.784	94.405	102.658	77.450	93.687	84.627	81.666	66.506	58.163
4	123.380	94.853	95.750	95.481	91.624	74.041	73.682	63.725	66.954
5	99.428	105.528	96.378	90.188	87.677	60.675	62.559	75.477	71.709
6	91.175	90.637	76.912	92.162	88.035	78.616	62.379	77.540	75.118
7	86.959	99.787	83.640	79.693	79.244	77.630	73.682	67.582	65.429
8	104.003	101.760	97.634	86.780	68.749	72.606	78.706	74.669	62.559
AVG	100	97.634	92.756	90.469	83.516	75.634	73.469	72.214	66.876
SD	11.747	4.667	9.361	8.862	8.311	7.052	7.292	5.374	6.676

Table 27 Cytotoxicity of pDNA/CS complexes on Huh7 cells

n	N/P ratio								
	control	4	8	12	16	24	32	48	64
1	87.566	87.429	98.527	108.666	96.746	102.911	103.048	97.568	84.826
2	99.349	101.267	97.979	101.952	100.445	98.253	94.965	96.609	79.209
3	101.404	112.502	94.417	113.461	92.773	96.609	96.883	97.431	82.086
4	105.926	100.582	101.267	87.840	102.226	94.828	96.472	94.965	87.566
5	100.445	105.378	101.130	100.719	89.485	94.417	86.196	81.812	83.593
6	105.926	96.472	100.171	97.157	99.760	100.993	84.963	84.552	83.319
7	100.171	97.020	94.280	94.280	95.513	97.842	87.018	80.305	79.757
8	99.212	86.470	100.582	94.006	100.445	94.006	82.223	86.744	80.305
AVG	100.000	98.390	98.544	99.760	97.174	97.482	91.471	89.998	82.583
SD	5.708	8.692	2.840	8.322	4.393	3.203	7.330	7.389	2.834

Table 28 Cytotoxicity of naked DNA and pDNA/PEI complexes on Huh7 cells

n	Samples		
	control	Naked DNA	pDNA/PEI (1:1 weight ratio)
1	119.214	96.677	89.923
2	116.682	119.148	79.195
3	104.128	110.708	73.931
4	104.022	94.672	74.720
5	95.899	79.058	78.406
6	99.591	83.911	71.693
7	89.147	101.635	104.599
8	71.317	119.465	63.467
AVG	100	100.659	79.492
SD	15.298	15.101	12.602

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European Journal of Pharmaceutics and Biopharmaceutics 70 (2008) 207–214
European Journal of Pharmaceutics and Biopharmaceutics
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Research paper

Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan for novel effective gene carriers

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Received 17 September 2007; accepted in revised form 21 April 2008
Available online 1 May 2008

Abstract

The objective of this study was to investigate the transfection efficiency of quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan, TM_{47}-Bz_{42}-CS, using the plasmid DNA encoding green fluorescent protein (pEGFP-C2) on human hepatoma cell lines (Huh7 cells), in comparison to quaternized chitosan (TM_{43}-CS) and chitosan (CS). Factors affecting the transfection efficiency, such as the carrier/DNA weight ratio, the pH of the culture medium, and the presence of serum, have been investigated. The results revealed that TM_{47}-Bz_{42}-CS was able to condense with pDNA. As illustrated by the agarose gel electrophoresis, the complete complexes of TM_{47}-Bz_{42}-CS/DNA were formed at a weight ratio of above 0.5, whereas those of TM_{43}-CS/DNA and CS/DNA were formed at a ratio of above 1. TM_{47}-Bz_{42}-CS showed superior transfection efficiency to TM_{43}-CS and CS at all weight ratios tested. Higher transfection efficiency and gene expression were observed when the carrier/DNA weight ratios increased. The highest transfection efficiency was found at a weight ratio of 8. The results indicated that the improved gene transfection was due to the hydrophobic group (*N*,*N*-dimethylaminobenzyl) substitution on CS, which promoted the interaction and condensation with DNA, as well as *N*-quaternization, which increased the CS water solubility. During cytotoxicity studies, it was found that high concentrations of TM_{47}-Bz_{42}-CS and TM_{43}-CS could decrease the Huh7 cell viability. In conclusion, this novel CS derivative, TM_{47}-Bz_{42}-CS, shows promising potential as a gene carrier by efficient DNA condensation and a mediated higher level of gene transfection in Huh7 cells.

Keywords: Trimethylaminobenzyl chitosan; Gene delivery; Huh7 cells; Transfection efficiency

1. Introduction

Gene delivery has been regarded as a powerful tool for curing disease by replacing defective genes, substituting missing genes, or silencing unwanted gene expression. The two main types of vectors used in gene therapy are viral and non-viral. Viral vectors are the most effective because of their evolutionary optimization for this purpose, but recently reported safety issues, such as random recombination, oncogenic potential, and immunogenicity, have set back the rapid development of viral vectors [1–2]. In light of these safety concerns, non-viral delivery systems have been developed. Among these, cationic liposomes are widely used for almost all animal cells because they have non-specific ionic interactions and low toxicity [3–5]. There are some limitations, however, because these liposomes are unstable when used for in vivo transfection. Therefore, many polymeric cationic systems, such as gelatin, polyethyleneimine (PEI), poly(L-lysines), tetraminofullerene, poly(L-histidine)-graft-poly(L-lysines), DEAE-

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doi:10.1016/j.ejpb.2008.04.022

dextrans, cationic dendrimers, and chitosan, have been studied for in vitro as well as in vivo applications [6–7].

Chitosan (CS) [(1 → 4) 2-amino-2-deoxy-β-D-glucan] is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) produced by the alkaline deacetylation of chitin. CS is a weak base with a pKa value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values but soluble in acidic mediums such as acetic acid, citric acid, glutamic acid, aspartic acid, hydrochloric acid, and lactic acid. CS has been used in drug delivery as an absorption enhancer [8] and as a vector for gene delivery. In addition, CS is biocompatible, biodegradable, and non-toxic; therefore, it has been proposed as a safer alternative to other non-viral vectors such as cationic lipids and cationic polymers [9–13]. At acidic pH, below their pKa, the primary amines in the chitosan backbone become positively charged. These protonated amines enable CS to bind to negatively charged DNA and condense DNA into particles. Moreover, CS has shown a capability to protect DNA from DNase I and II degradation [14]. Formulation parameters such as molecular weight (M_W), degree of deacetylation (DD), *N/P* ratio (ratio of positively charged chitosan to negatively charged DNA), and pH of the transfection medium were found to affect the transfection efficiency of CS/DNA complexes [15–18].

The main drawback of CS is its poor water solubility at physiological pH, and its low transfection efficiency. Several CS derivatives have been synthesized in the last few years to obtain a modified carrier with altered physicochemical characteristics. Using modified CS, such as glycol CS or PEGylated CS [19–20], low molecular weight soluble CS [9], and quaternized CS [21], could be possible ways to circumvent the known solubility issues. To improve gene transfection, chemically modified CS, such as quaternized CS [21], urocanic acid-modified CS [22], galactosylated CS [23], deoxycholic acid CS oligosaccharide nanoparticle [24], and thiolate CS [25], was reported.

Although many researches have synthesized CS derivatives as alternatives for gene carriers, few were successful in increasing the transfection efficiency. In this study, the water-soluble CS derivatives, which favor the interaction with pDNA, have been synthesized and evaluated for their in vitro transfection efficiency and cytotoxicity. CS was substituted with an *N,N*-dimethylbenzyl group to provide the hydrophobic moiety for the improved hydrophobic interaction with pDNA, and it was quaternized to render CS soluble. A number of variables that influence the transfection efficiency, such as carrier/DNA weight ratio, particle size, ζ-potential, morphology, and the pH of the culture medium and serum, were investigated.

2. Materials and methods

2.1. Materials

CS was purchased from Seafresh Chitosan Lab (Bangkok, Thailand) with a M_W of 267 kDa and a 94% degree of deacetylation. Sodium cyanoborohydride and polyethylenimine (PEI), M_W 25 kDa, were purchased from Aldrich (Milwaukee, USA). Iodomethane, 4-*N,N*-dimethylaminobenzaldehyde, sodium iodide, and 1-methyl-2-pyrrolidone were purchased from Fluka (Deisenhofen, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Trypsin–EDTA, penicillin–streptomycin antibiotics, and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen (Grand Island, NY, USA). The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, Palo Alto, USA. The λ*Hin*dIII were obtained from Promega (Madison, WI USA). Huh7 (Human hepatocellular carcinoma) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

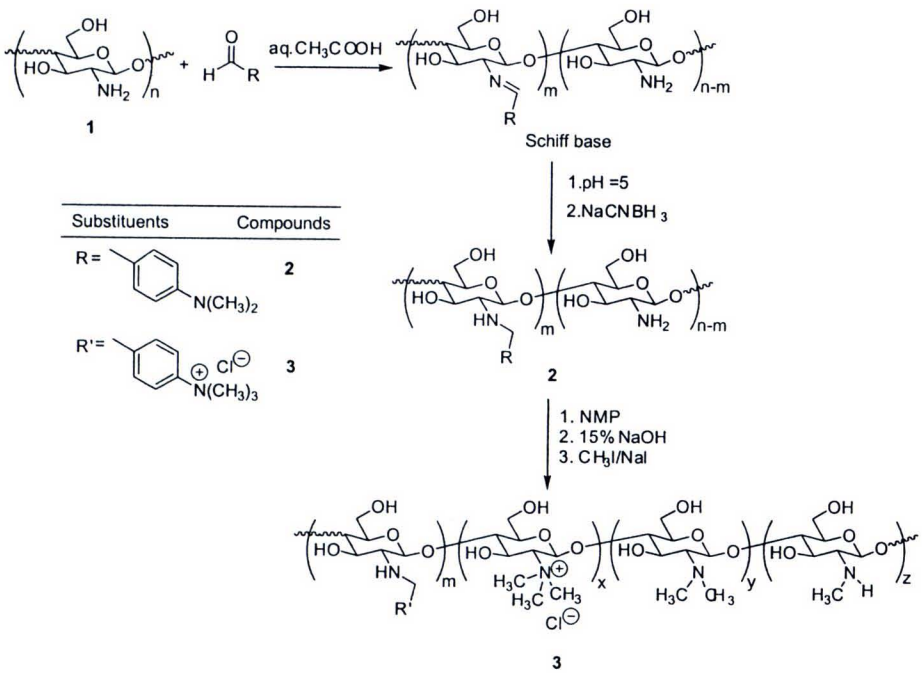
2.2. Synthesis of methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS)

2.2.1. Synthesis of N-(4-N,N-dimethylaminobenzyl) chitosan (DM-Bz-CS)

DM-Bz-CS 2 was prepared as previously reported [26]. Briefly, 1.00 g of chitosan 1 (6.11 meq/GlcN) was dissolved in 0.2 M acetic acid (pH 4, 70 mL). The solution was diluted with ethanol (70 mL), and an aromatic aldehyde (4-*N,N*-dimethylamino benzaldehyde (3.0 meq/GlcN) was added and stirred at room temperature for 1 h. The pH of the solution was adjusted to 5 with 1 N NaOH. Subsequently, 1.54 g of $NaCNBH_3$ was added and stirred at room temperature for 24 h, followed by a pH adjustment to 7 with 15% (w/v) NaOH. The reaction mixture was then dialyzed in distilled water and freeze-dried to give powder 2 (Scheme 1).

2.2.2. Synthesis of trimethylated CS and CS derivatives

Compound 1 or **2** (0.50 g) was dispersed in 25 mL of *N*-methyl pyrrolidone (NMP) for 12 h at room temperature. Then, 1.5 g of 15% sodium iodide and 5% w/v NaOH (3.0 mL) were added and stirred at 50 °C for 15 min. Subsequently, 1 mL of methyl iodide was added in three portions at 4 h intervals and stirred for 12 h at 50 °C. The reaction mixture appeared yellow and clear. The obtained compounds were purified by precipitation in 300 mL of acetone. The precipitate was dissolved in a 15% (w/v) NaCl solution to replace the iodide ion with chloride ion. The suspension was dialyzed with deionized water for 3 days to remove inorganic materials. The dialyzed solution was then concentrated under vacuum on a rotary evaporator and then precipitated in acetone (100 mL). The pure compounds (TM-CS and TM-Bz-CS 3) were collected and dried overnight at room temperature under a stream of nitrogen (Scheme 1). TM-CS, after being obtained from the first methylation, was subjected to repeated methylation to yield a higher tri-methyl substitution.



Scheme 1. Synthesis of methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan.

2.3. Characterization

FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared (FT-IR) spectrometer, and all samples were prepared as potassium bromide pellets. The ^{1}H and ^{13}C NMR spectra were measured on a Mercury Varian 300 MHz spectrometer. All measurements were performed at 300 K, using a pulse accumulation of 64 scans and the LB parameter of 0.30 Hz. D_2O/CF_3COOD (1% (v/v)) and D_2O were used as solvents for 10 mg of chitosan and its derivatives, respectively.

2.4. Plasmid preparation

The pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA). The DNA concentration was quantified by the measurement of UV absorbance at 260 nm using a GeneRay UV Photometer (Biometra®). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate–EDTA (TAE) buffer at pH 8.0, using λDNA/*Hin*dIII as a DNA marker.

2.5. Preparation and characterization of CS derivatives / DNA complexes

The CS derivatives/DNA complexes were prepared at various carrier/DNA weight ratios by adding the DNA solution to the CS derivative solution. The mixture was gently mixed using a pipette for 3–5 s to initiate complex formation and left for 15 min at room temperature. The complex formation was confirmed by electrophoresis. Agarose gels were prepared with 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/mL). The electrophoresis was carried out for 60 min at 100 V. A CS derivatives/DNA complex sample (15 μL) containing 1 μg of DNA was loaded in the well.

2.6. Size and ζ potential measurements

The particle size and surface charge of CS derivatives/DNA complexes were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water, which was passed through a 0.22 μm membrane filter prior to use. All samples were measured in triplicate.

2.7. Morphology

The morphology of the polyplexes was analyzed by atomic force microscope (AFM). Complexes of plasmid DNA and TM_{47}-Bz_{42}-CS were prepared at the weight ratio of 8. The complexes were diluted with distilled water and applied to freshly cleaved mica. After allowed to bind for 20–120 s, the mica was then rinsed twice with 100 mL distilled water and the excess liquid wicked away at the mica edge with a tissue. The mica was then blown until completely dry with a stream of nitrogen gas and put into a vacuum desiccator until it could be imaged by AFM.

2.8. In vitro transfection of CS/DNA complexes in Hun 7 cells

Huh7 cells were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 mL of growth medium (DMEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/mL penicillin and 100 μg/mL streptomycin). The cells were grown under a humidified atmosphere (5% CO_2, 95% air, 37 °C) for 24 h. Prior to transfection, the medium was removed, and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). The cells were incubated with 0.5 mL of the CS derivatives/DNA complexes at various *N/P* ratios containing 1 μg of pDNA for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes were used as controls. After transfection, the cells were washed twice with PBS and grown in a culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

2.9. Evaluation of cell viability

The evaluation of cytotoxicity was performed by the MTT assay. Huh7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μL of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to transfection, the medium was removed, and the cells were rinsed with PBS and then supplied with the CS derivatives/DNA complexes in the same concentrations as in the in vitro transfection experiment. After treatment, solutions of the CS derivatives/DNA complexes were removed. Finally, the cells were incubated with 100 μL MTT containing medium (1 mg/mL) for 4 h. The medium was subsequently removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μL DMSO per well. The relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). The viability of non-treated control cells was arbitrarily defined as 100%.

2.10. Statistical analysis

The statistical significance of differences in the transfection efficiency and cell viability was examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of CS derivatives

As previously reported, DM-Bz-CS 2 was synthesized by reductive amination of the corresponding Schiff base intermediates (Scheme 1) [26]. The extent of *N*-substitution (ES), determined by ^{1}H NMR, varied due to the different ratio of aldehydes to GlcN in CS. The FT-IR spectra of DM-Bz-CS were similar to that of CS, except that additional absorption bands at wavenumbers 1605, 1526, and 811 cm^{-1} were observed. These bands were assigned to the C=C stretching and C–H deformation (out of plane) of the aromatic group. The ^{1}H NMR spectrum of DM-Bz-CS exhibited a broad singlet, δ 7.5 ppm, in the aromatic region and another singlet at δ 3.1 ppm assigned to *N,N*-dimethyl protons. The ^{1}H NMR spectra of TM-Bz-CS were similar to that of the corresponding TM-CS, except for additional signals at δ 3.5 ppm, which corresponded to the *N,N,N*-trimethyl protons on the benzyl substituent.

In this study, the quaternization of DM-Bz-CS 2 was based on a nucleophilic substitution of the primary amino group on the C-2 position of CS using procedures that were slightly modified from the method previously described [27]. The quaternization of DM-Bz-CS 2 with methyl iodide yielded TM-Bz-CS 3, which could occur at both the aromatic substituent and primary amino group of the GlcN of chitosan. The results clearly demonstrated that the *N,N*-dimethylamino groups of DM-Bz-CS 2 were more reactive than the primary amino groups of chitosan, which were completely quaternized, giving DQ_{Ar} values equal to the corresponding ES's (Table 1). In addition to *N,N,N*-trimethylation, *N,N*-dimethylation, and *N*-monomethylation at the primary amino group of GlcN of chitosan were also observed. In this case, *O*-methylation at the GlcN of CS is also observed by the appearance of small signal at δ 3.3 and 3.4 ppm.

TM-CS. FT-IR (KBr): ν 3444, 1475, 1107, 1071, and 1057 cm^{-1}. ^{1}H NMR (D_2O): δ (ppm) 5.42 (br s; 1H H1, H1′), 4.40–3.01 (br o; 23H –NH–CH_2–, H2, H3, H4, H5, H6 and H6′, s; OCH_3, br s; $N^+(CH_3)_3$), 2.71 (br m; 6H $N(CH_3)_2$), 2.31 (s; 3H $NHCH_3$), 1.97 (s; 3H $NHCOCH_3$). ^{13}C NMR (D_2O): δ (ppm) 96.55 (C1), 77.62 (C4), 74.74 (C5), 68.85 (C5), 60.0–55.5 (C2 and C6), 54.43 ($N^+(CH_3)_3$), 42.71 ($N(CH_3)_2$).

TM-Bz-CS. FT-IR (KBr): ν 3442, 1559, 1475, 1147, 1104, 1059, and 850 cm^{-1}. ^{1}H NMR (D_2O): δ (ppm) 7.75–7.50 (dd; 4H Ph), 5.40, 4.96 (s; 2H H1, H1′), 4.42–3.13 (br m; 32H –NH–CH_2–, H2, H3, H4, H5, H6 and H6′, br s; $N^+(CH_3)_3$ Ph, s; OCH_3, br s; $N^+(CH_3)_3$), 2.71 (br m; 6H $N(CH_3)_2$), 2.31 (s; 3H $NHCH_3$), 1.97 (s; 3H $NHCOCH_3$). ^{13}C NMR (D_2O): δ (ppm) 145.52, 141.53, 130.98, 119.56 (C-Ph), 96.55 (C1), 77.13–58.67 (C2, C3, C4, C5, and C6), 56.98 ($N^+(CH_3)_3$ Ph), 53.88 ($N^+(CH_3)_3$), 41.77 ($N(CH_3)_2$), 36.30 (NCH_3).

3.2. Characterization of CS derivatives/DNA complexes

To determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between either CS or CS derivatives and DNA at different CS concentrations. The formation of complexes between CS derivatives and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. By varying the concentration

Table 1
Quaternization of chitosan and *N*-benzyl chitosan derivatives

Samples	ES (%)	DQ_T (%)		DM-CS (%)	M-CS (%)	Recovery (%)
		DQ_{Ar} (%)	DQ_{CS} (%)			
TM_{43}-CS	–	–	43	48	–	80
TM_{47}-Bz_{42}-CS	42	42	5	Trace	7	78

ES is the extent of *N*-substitution; DQ_{Ar} is degree of quaternization at aromatic substituents; DQ_{CS} is degree of quaternization; DM-CS is *N,N*-dimethylation of GlcN of CS; M-CS is *N*-monomethylation GlcN of CS; Recovery (%) is [weight of product (g)/weight of starting reactant (g)] × 100.

of CS and fixing the DNA concentration, the weight ratios of positively charged CS (due to amine groups) to negatively charged DNA (due to the phosphate groups), referred to as weight ratios of the particle formulations, were varied (Fig. 1; lanes 3–8, CS/DNA complexes with weight ratio of 0.5, 1, 2, 4, 6, and 8). As shown in Fig. 1, TM_{47}-Bz_{42}-CS showed different gene condensation patterns. In the case of TM_{47}-Bz_{42}-CS (Fig. 1a), complete complexes were formed at carrier/DNA weight ratios above 0.5. In contrast, DNA complexes with TM_{43}-CS or CS had carrier/DNA weight ratios above 1. These results revealed that the hydrophobic interactions between trimethylaminobenzyl moieties of TM_{47}-Bz_{42}-CS and charge-neutralized DNA segments were possibly responsible for the enhanced gene condensation. The hydrophobic moiety-dependent gene condensation capacity phenomenon is in close agreement with the previously reported results [24].

The particle size and ζ potential were plotted against weight ratios of the formulated CS derivatives/DNA complexes (Fig. 2). The particle size of the TM_{47}-Bz_{42}-CS/DNA complexes increased with the increasing weight ratio from 0.5 to 1 and decreased to a constant value in the range of 200 to 300 nm after a weight ratio of 2 (Fig. 2a). At an *N/P* ratio of 1, TM_{47}-Bz_{42}-CS/DNA complexes had the largest particle size. An initial negative value of the ζ potential was observed at a low charge ratio of 0.5. At weight ratios between 0.5 and 1, the ζ potential was approximately neutral, but the particle size of the TM_{43}-CS/DNA complexes slightly increased with an increasing weight ratio from 1 to 2 and decreased to a constant value in the range of 176–300 nm after a weight ratio of 2 (Fig. 2b). The ζ potential of the complexes was found to increase with an increase in the weight ratios of CS derivatives due to the higher density of protonated amines in the CS backbone. A similar result was observed in CS (Fig. 2c).

The morphological examination of the TM_{47}-Bz_{42}-CS/DNA complexes at a weight ratio of 8 was performed by AFM. The AFM images revealed that the complexes were spherical with nano size.

3.3. In vitro transfection

The achievement of high gene transfection efficiency is a final goal for the development of novel gene carriers. To investigate the CS derivative-mediated gene transfection efficiencies, an in vitro gene transfection assay was performed with human hepatoma cell lines (Huh7 cells) using the pEGFP-C2 plasmid encoding green fluorescent protein (GFP). CS derivatives/DNA complexes were formulated at various weight ratios [2,4,6,8,12,16,24,32] to investigate the optimal conditions for gene transfection. Polyethylenimine (PEI, 25 kDa), complexed with DNA at the weight ratio of 1 was used as a positive control. In all studies, no transfection was seen in the control (cells without complexes) and naked DNA. As shown in Fig. 3 (at pH 7.4), the gene transfection efficiencies were significantly influenced by the carrier/DNA ratios. By increasing the ratios, the transfection efficiencies reached the highest values, with a decrease by further increment of the ratios. Among the carriers, TM_{47}-Bz_{42}-CS showed the highest transfection efficiency (Fig. 3a). Its transfection efficiency (carrier/DNA weight ratio of 8) was 6 and 140 times higher in gene transfection than that of TM_{43}-CS (Fig. 3b) and CS (Fig. 3c), respectively. These results revealed that not only the trimethyl groups but also the hydrophobic groups (trimethylaminobenzyl moieties) affected the gene transfection efficiency. Previous studies reported that the transfection efficiency of CS was dependent on pH and serum. Chitosan-mediated high gene transfection was observed at pH values below 6.5 [28]. Cationic liposome-associated gene

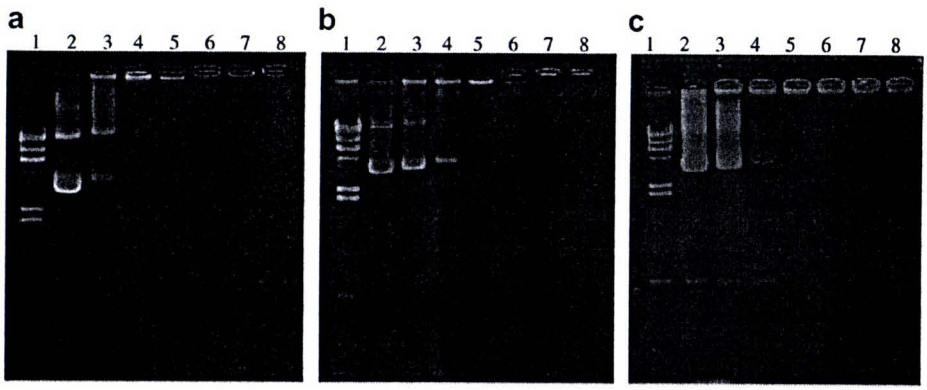


Fig. 1. Gel retarding analysis of CS derivatives/DNA complexes formulated with (a) TM_{47}-Bz_{42}-CS, (b) TM_{43}-CS, and (c) CS. Lane 1, DNA marker; lane 2 pEGFP-C2 plasmid; lanes 3–8, CS derivatives/DNA complexes at weight ratios of 0.5, 1, 2, 4, 6, and 8, respectively.

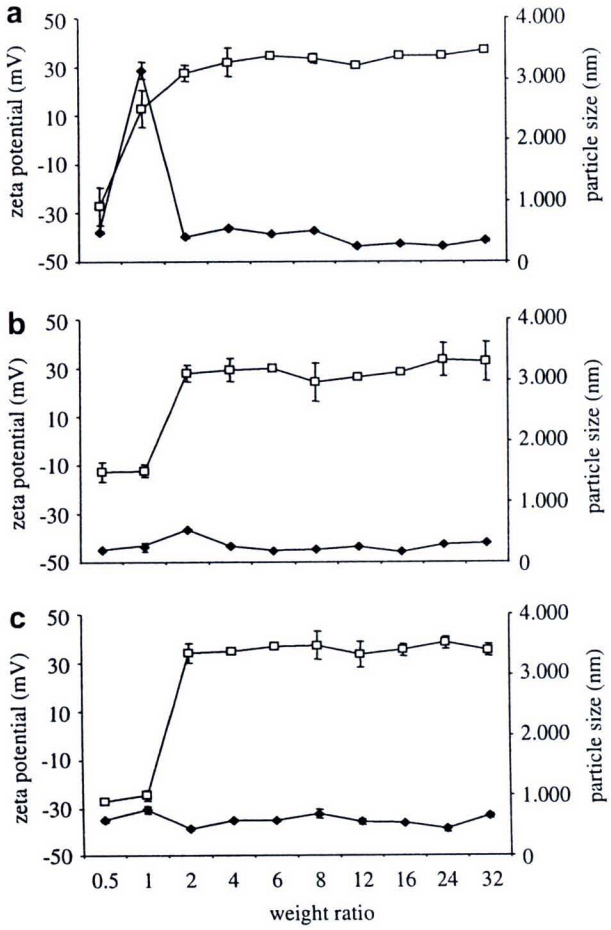


Fig. 2. ζ Potential (□) and particle size (♦) at varying weight ratios of CS derivatives/DNA complexes formulated with (a) TM_{47}-Bz_{42}-CS, (b) TM_{43}-CS, and (c) CS. Each value represents means ± SD of three measurements.

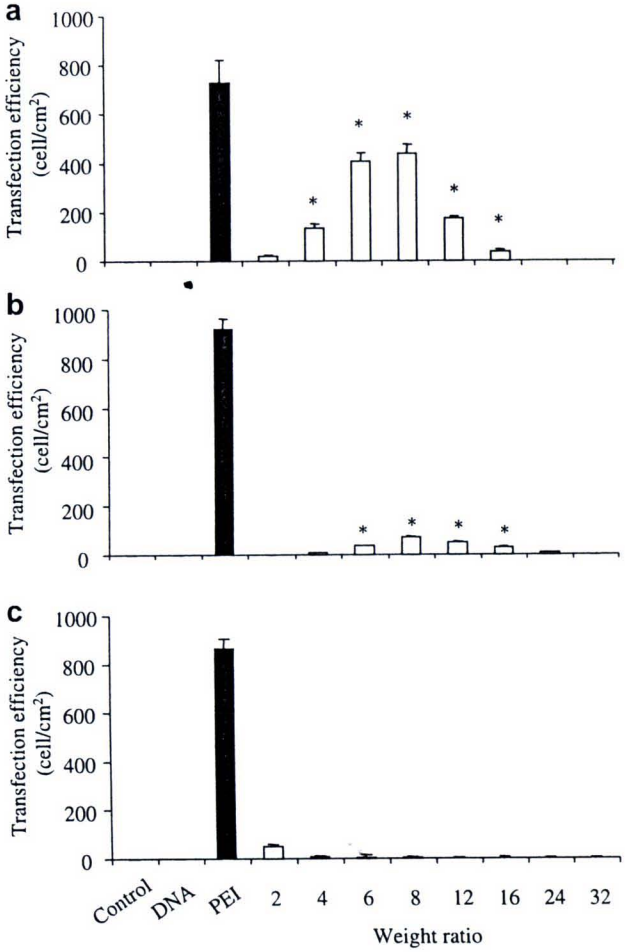


Fig. 3. Transfection efficiencies of CS derivatives/DNA complexes formulated with (a) TM_{47}-Bz_{42}-CS, (b) TM_{43}-CS, and (c) CS in Huh7 cells. Each value represents means ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

expression was inhibited by serum, while CS showed resistance to serum [15]. As shown in Fig. 4, TM_{47}-Bz_{42}-CS and CS associated gene expression was inhibited by the presence of 10% serum. Changes in pH dramatically affected the transfection efficiency of CS by causing a decrease in efficiency with an increase in pH from 6.5 to 7.4. In contrast, TM_{47}-Bz_{42}-CS was not influenced by pH.

Partially quaternized polymers have been previously referred to as promising transfection agents. Cationic polymers containing quaternary charged trimethylamino ethylmethacrylate (TMAEM) copolymerized with hydrophilic-(2-hydroxypropyl) methacrylates (HPMA) were found to condense DNA and transfect 293 cells [29]. Trimethylated chitosan oligomers are promising agents for DNA condensation and promote the transfection efficiency on COS-1 and Caco-2 cells [21] and COS-7 and MCF-7 cells [30]. This permanent positive charge of the trimethylated chitosan is a key factor for the condensation and protection of DNA. The introduction of an *N*-benzyl group into the CS polymer backbone enhances the hydrophobicity, which improves the hydrophobic interactions between the poly-

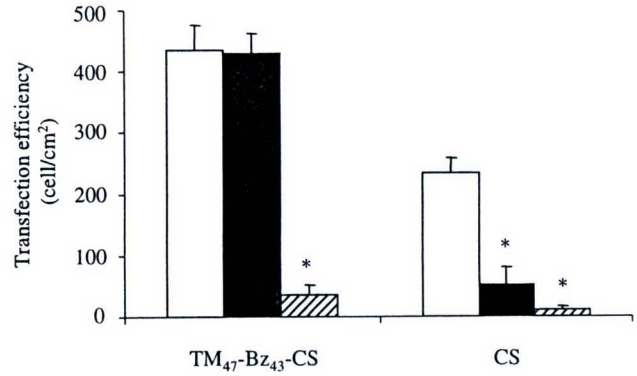


Fig. 4. Effect of pH medium and serum at (□) pH 7.4 without serum (■) pH 6.2 without serum and (▨) pH 7.4 with 10% serum, on transfection efficiencies of TM_{47}-Bz_{42}-CS/DNA complexes and CS/DNA complexes at a weight ratio of 8 in Huh7 cells. Each value represents means ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

mer and DNA, and DNA condensation. In addition, it improves hydrophobic interactions with the cell membrane [24,31]. These help the water-soluble CS to be an efficient vector. As reported previously, hydrophobically modified cationic polymers or cationic lipids have shown high gene transfection capability as an optimal substitution by increasing cell membrane/carrier interactions or the destabilization of the cell membranes [32]. Although the exact mechanism of TM_{47}-Bz_{42}-CS mediated efficient gene delivery and the optimal degree of trimethylated and hydrophobic groups (*N*-benzyl group) must be studied further, our first results showed that TM_{47}-Bz_{42}-CS could be a potential candidate for non-viral gene carriers.

3.4. Effect of CS derivatives/DNA complexes on cell viability

One of the major requirements for cationic polymer vectors for gene delivery is low cytotoxicity. It has been reported that CS and CS derivatives were less toxic than other cationic polymers, such as poly-lysine and polyethyleneimine, in vitro and in vivo [21]. Various chitosans and chitosan derivatives have been reported for gene delivery, but the toxicity of those chitosans varied depending on the type of cells and derivatives studied. Therefore, the cytotoxicity study of the CS derivatives/DNA complexes was performed in Huh7 cells. Fig. 5 shows the effect of TM_{47}-Bz_{42}-CS/DNA (Fig. 5a), TM_{43}-CS/DNA (Fig. 5b), and CS/DNA complexes (Fig. 5c) on cell viability. When Huh7 cells were incubated with 1 μg of naked DNA, the cell viability remained almost the same as that seen in non-transfected cells used as a control (data not shown). There was a significant decrease in cell viability when Huh7 cells were incubated with various weight ratios of both TM_{47}-Bz_{42}-CS/DNA and TM_{43}-CS/DNA complexes compared to that seen for CS/DNA complexes. Though their average cell viability decreased when the carrier/DNA weight ratios increased, the viability was over 80% at a weight ratio of 8, from which the highest transfection efficiency was obtained. Therefore, from this study, TM_{47}-Bz_{42}-CS has clearly been proven to be safe.

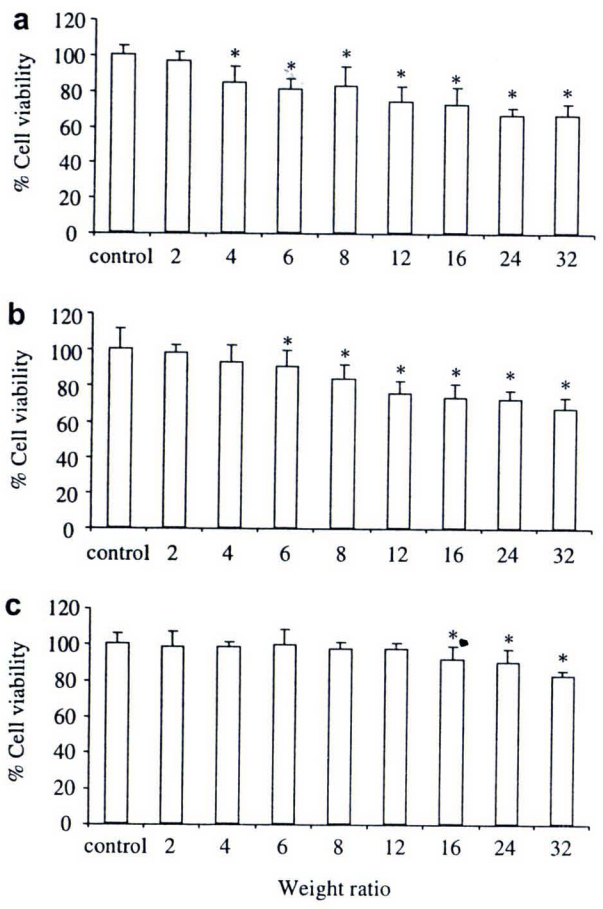


Fig. 5. Cell viability of CS derivatives/DNA complexes formulated with (a) TM_{47}-Bz_{42}-CS, (b) TM_{43}-CS and (c) CS in Huh7 cells. Each value represents means ± SD of six wells. Difference values * were statistically significant ($p < 0.05$).

4. Conclusion

In this study, a novel water-soluble chitosan derivative (TM_{47}-Bz_{42}-CS) was successfully synthesized with the goal of improving the transfection efficiency. This was done by chemically modifying chitosan with a hydrophobic moiety of *N,N*-dimethylaminobenzyl and increasing the solubility by quaternization. The results of this study suggest that TM_{47}-Bz_{42}-CS is safe and exhibits significantly improved gene delivery potential in vitro.

Acknowledgments

The authors are grateful for the financial support provided by the National Nanotechnology Center (NANOTEC), Thailand (Grant No.: NN-B-22-b3-17-49-07), the Commission of Higher Education (Thailand), and The Thailand Research Funds (Grant No.: DBG518005).

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International Journal of Pharmaceutics 364 (2008) 127–134

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Methylated *N*-(4-pyridinylmethyl) chitosan as a novel effective safe gene carrier

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ARTICLE INFO

Article history:
Received 22 May 2008
Received in revised form 9 July 2008
Accepted 1 August 2008
Available online 15 August 2008

Keywords:
Methylated *N*-(4-pyridinylmethyl) chitosan
Gene delivery
Huh 7 cells
Transfection efficiency

ABSTRACT

The objective of this study was to study the transfection efficiency of quaternized *N*-(4-pyridinylmethyl) chitosan; TM-Py-CS, using the pDNA encoding green fluorescent protein (pEGFP-C2) on human hepatoma cell lines (Huh 7 cells). The factors affecting the transfection efficiency, e.g. degree of quaternization (DQ), the extent of *N*-pyridinylmethyl substitution (ES) and weight ratio, have been investigated. The results revealed that TM-Py-CS was able to condense with pDNA. Illustrated by agarose gel electrophoresis, complete complexes of $TM_{69}Py_{62}CS$/DNA were formed at weight ratio above 1.1, whereas those of $TM_{53}Py_{40}CS$/DNA and $TM_{52}Py_{13}CS$/DNA were above 1.8 and 8, respectively. $TM_{69}Py_{62}CS$ showed superior transfection efficiency to $TM_{53}Py_{40}CS$, $TM_{52}Py_{13}CS$, $TM_{65}CS$ and $TM_{43}CS$ at all weight ratios tested. The highest transfection efficiency of $TM_{69}Py_{62}CS$/DNA complexes was found at weight ratio of 4. The results indicated that the improved gene transfection was possibly due to 4-pyridinylmethyl substitution on CS which promoted the interaction and condensation with DNA as well as *N*-quaternization which increased CS water solubility. In cytotoxicity studies, high concentration of TM-Py-CS and TM-CS could decrease the Huh 7 cell viability. In conclusion, this novel CS derivative, $TM_{69}Py_{62}CS$, showed promising potential as a gene carrier by efficient DNA condensation and mediated higher level of gene transfection.

1. Introduction

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. The main objective in gene therapy is successful in vivo transfer of the genetic materials to the targeted tissues in order to replace defective genes, substitute missing genes or silence unwanted gene expression (Zhang et al., 2004). However, naked therapeutic genes are rapidly degraded by nucleases and show poor cellular uptake, so the development of safe and efficient gene carriers is one of the prerequisites for the success of gene therapy (Rolland, 2005). The development of new delivery systems for the administration of gene therapeutics is a field of great interest. One approach is a non-viral delivery system based on supramolecular assembly. Cationic lipids (Lonez et al., 2008) and cationic polymers (Garnett, 1999) have been employed as non-viral gene transfer agents. These cationic substances form complexes with anionic DNA by electrostatic interaction. The resultant cationic DNA complexes are taken up by cells through electrostatic interaction because the cell surface is negatively charged. Transfection efficiencies of these complexes have been investigated in vivo and in vitro. However, before putting these complexes to practical use, there are several problems to overcome: insufficient transfection efficiency; strong cytotoxicity; and inhibition by serum. Biodegradable and biocompatible polymers are suitable for human use and can be prepared as particle complexes of various sizes. Particles with suitable sizes and charges can enter mammalian cells by several routes, such as pinocytosis, phagocytosis, receptor-mediated uptake, etc. and this may improve the chances of cellular entry.

Chitosan (CS) [a (1 → 4) 2-amino-2-deoxy-β-D-glucan] is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine produced by alkaline deacetylation of chitin. Chitosan is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values. However, CS is soluble in acidic medium such as acetic acid, citric acid, glutamic acid, aspartic acid, hydrochloric acid, lactic acid, etc. Chitosan has been used in drug delivery as an absorption enhancer and as a vector for gene delivery. In addition, chitosan is biocompatible, biodegradable and nontoxic; therefore, it has been proposed as a safer alternative to other non-viral vectors such as cationic lipids and cationic polymers (Lee et al., 2001; Ishii et al., 2001; Kiang et al., 2004; Weecharangsan et al., 2006). At acidic pH, below pK_a, the primary amines in the chitosan backbone become positively

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doi:10.1016/j.ijpharm.2008.08.003

charged. These protonated amines enable chitosan to bind to negatively charged DNA and condense it into particles. Chitosan has shown promise to protect DNA from DNase I&II degradation (Huang et al., 2005). Formulation parameters such as molecular weight (MW), degree of deacetylation (DD), N/P ratio (ratio of positively charged chitosan to negatively charged DNA), and pH of transfection medium were found to affect the transfection efficiency of CS/DNA complexes (Sato et al., 2001; Romóren et al., 2003; Lavertu et al., 2006, Weecharangsan et al., 2008).

The main drawback of CS is insoluble at physiological pH and low transfection efficiency. Several CS derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics. To address the solubility issue, modified CS such as glycol CS or PEGylated CS (Yoo et al., 2005), low molecular weight soluble CS (Lee et al., 2001) and quaternized CS (Thanou et al., 2002) could be another possible way to escape the solubility issues. To improve gene transfection, chemically modified CS, such as quaternized CS (Thanou et al., 2002), urocanic acid-modified CS (Kim et al., 2003), galactosylated CS (Gao et al., 2005), deoxycholic acid CS oligosaccharide nanoparticle (Chae et al., 2005), and thiolated CS (Lee et al., 2007) were synthesized in order to obtain modified carrier with altered physicochemical characteristics. Although many researchers synthesized CS derivatives as alternative for gene carrier, a few were successful in increased transfection efficiency. Recently, our research group have successfully synthesized the novel water-soluble CS derivatives namely methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan. This CS derivatives showed elevated potential as gene carrier by efficient DNA condensation and mediated higher level of gene transfection with negligible cytotoxicity in Huh 7 cells (Rojanarata et al., 2008). However, the chemical structure requirements related to the ability of synthetic chitosan derivatives to improve the transfection efficiency remain largely obscure. The novel derivatives with desired properties are of interest and deserve the intensive investigation. The pyridinium surfactant have been developed and showed high transfection efficiency and nontoxic in vitro gene delivery (van der Woude et al., 1997). Therefore, the pyridinium group was introduced into the chitosan in order to enhance the transfection efficiency and lower cytotoxicity. In this study, the novel water-soluble CS derivatives, methylation of chitosan containing pyridinylmethyl substituent have been synthesized, and evaluated for their in vitro transfection efficiency and cytotoxicity. CS was substituted with *N*-pyridinylmethyl group to produce hydrophobicity for improved hydrophobic interaction with pDNA and it was quaternized to produce soluble CS. A number of variables that influenced transfection efficiency such as extent of *N*-pyridinylmethyl substitution (ES), degree of quaternization (DQ) and weight ratio were determined. The physical properties of the complexes were investigated. Their transfection efficiencies and cytotoxicity in human hepatocellular carcinoma cells (Huh 7 cells) were evaluated.

2. Materials and methods

2.1. Materials

Chitosan, CS, (MW, of 276 kDa), was purchased from Seafresh Chitosan (lab) Co., Ltd. in Thailand. 4-Pyridinecarboxaldehyde and iodomethane were purchased from Riedal-deHaen, Seelze, Germany. Sodium cyanoborohydride and polyethyleneimine (PEI), MW 25 kDa, were purchased from Aldrich, Germany. 1-Methyl-2-pyrrolidone (NMP) was purchased from Fluka, Germany, and all other reagents were distilled before use. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM), Trypsin-EDTA, penicillin–streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen, USA. The pEGFP-C2 Plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, USA. The λ*Hin*dIII were obtained from Promega, USA. Huh7 (Human hepatocellular carcinoma) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

2.2. Synthesis of N-pyridinylmethyl chitosans (Py-CS)

The synthesis protocol for *N*-pyridinylmethyl chitosans (Py-CS) was shown in Scheme 1. In brief, chitosan was deacetylated to obtain 94% degree of deacetylation (DD; determined by ^{1}H NMR) (Lavertu et al., 2003). 1.00 g of chitosan was dissolved in 70 mL of 1% acetic acid solution. The solution was diluted with 70 mL ethanol, and then 0.58–1.16 mL of 4-pyridinecarboxaldehyde was added to the solution. The reaction mixture was stirred at room temperature for 24 h. At this point the pH of the solution was adjusted to 5 by adding 15% NaOH. Then, 1.54 g of $NaCNBH_3$ was added, and the resulting solution was allowed to stir at room temperature for 24 h, followed by adjusting the pH to 7 with 15% NaOH. The aqueous solution was dialyzed against de-ionized (DI) water using dialysis tubing with MW cut-off of 12,000–14,000 g/mol (Aldrich, Germany) for 3 days, followed by freeze drying.

2.3. Methylation of chitosans (TM-CS) and N-(4-pyridinylmethyl) chitosans (TM-Py-CS)

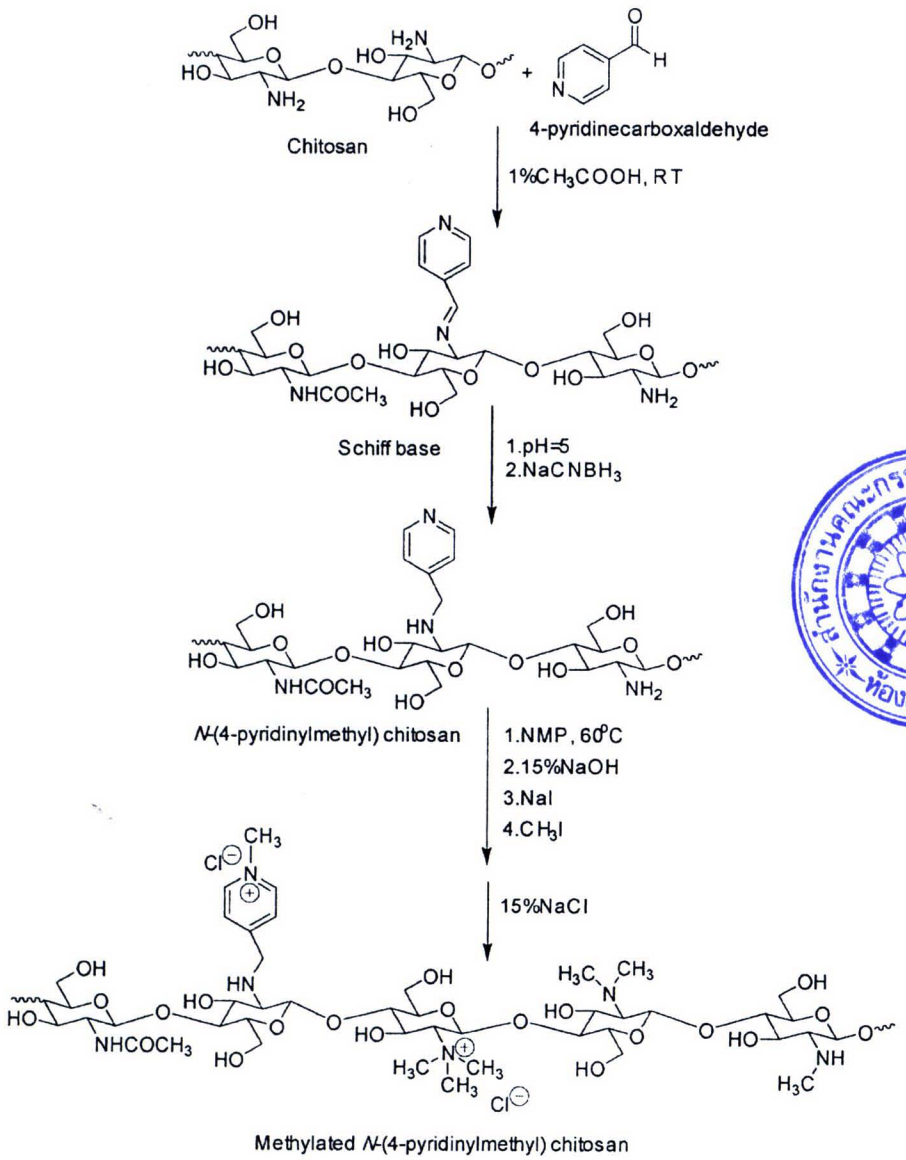
Either chitosans (CS) or *N*-(4-pyridinylmethyl) chitosans (Py-CS) was methylated in accordance to a reported procedure (Scheme 1) in order to enhance the solubility in water (Rodrigues et al., 1998; Sajomsang et al., 2008). In brief, 1.00 g of either CS or Py-CS was dispersed in 50 mL of 1-methyl-2-pyrrolidone (NMP) at room temperature and the mixture was stirred for 12 h. Then 8 mL of 15% aqueous NaOH was dropped slowly in the solution (for high degree of quaternization of CS, 20% aqueous NaOH was used instead of 15% aqueous NaOH). Sodium iodide (3.0 g) was added and the mixture was stirred at 60 °C for 15 min. Subsequently, 3, 3 and 2 mL of iodomethane was added three times, respectively every 4 h and the mixture was stirred at 60 °C for 12 h. After methylation, methylated chitosan and its derivatives were precipitated in 300 mL of acetone. The precipitate was dissolved in 15% NaCl solution in order to replace the iodide counter-ion with a chloride counter-ion. The suspension was dialyzed with de-ionized water for 3 days to remove inorganic materials and then freeze-dried.

2.4. Characterizations of chitosan and its derivatives

The chemical structures of chitosan and its derivatives were confirmed by Fourier Transform Infrared (FT-IR) spectra (Nicolet Impact 410 FT-IR spectrometer) and all samples were prepared as potassium bromide pellets. The degree of deacetylation (DD), the extent of *N*-substitution (ES) and degree of quaternization (DQ) were calculated based on ^{1}H NMR spectra, which were obtained using a Bruker AVANCE 500 MHz Spectrometer. All measurements were performed at 300 K, using pulse accumulating of 64 scans and the LB parameter of 0.30 Hz. 1% of D_2O/CF_3COOD and D_2O were used as the solvents for dissolving 5 mg of CS, Py-CS and TM-Py-CS, respectively.

2.5. Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit



Scheme 1. Synthesis of methylated *N*-(4-pyridinylmethyl) chitosan.

(Qiagen, Santa Clarita, CA, USA). DNA concentration was quantified by measurement of UV absorbance at 260 nm and 280 nm using a GeneRay UV Photometer (Biometra® λ260/280 nm). The purity of the plasmid was checked by gel electrophoresis (1% agarose gel) in Tris acetate-EDTA (TAE) buffer, pH 8.0 using λDNA/*Hin*dIII as a DNA marker.

2.6. Preparation and characterization of CS derivatives/DNA complexes

The CS derivatives/DNA complexes were prepared at various charges or weight ratios by adding the DNA solution to the CS derivatives solution. The mixture was gently mixed by pipetting up and down for 3–5 s to initiate complex formation. After left for 15 min at room temperature for the complete reaction, the complex formation was confirmed by agarose gel electrophoresis, prepared from 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/mL). The electrophoresis was carried out for 60 min at 100 V. The volume of the sample loaded in the well was 15 μL of CS derivatives/DNA complex containing 1 μg of DNA.

2.7. Size and zeta potential measurements

The particle size and surface charge of CS derivatives/DNA complexes were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water and the solution was passed through 0.22 μm membrane filter before used. All samples were measured in triplicate.

2.8. In vitro transfection CS/DNA complexes in Huh 7 cells

Huh 7 cells were seeded 24 h into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 mL of growth medium (DMEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential

amino acid solution, 100 U/mL penicillin and 100 μg/mL streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air and 37 °C) for 24 h. Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). The cells were incubated with 0.5 mL of the CS derivatives/DNA complexes at various weight ratios containing 1 μg of pDNA for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes were used as controls. After transfection, the cells were washed with PBS twice and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

2.9. Evaluation of cell viability

Cytotoxicity of CS derivatives/DNA complexes was evaluated by the MTT assay. Huh 7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μL of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to transfection, the medium was removed and the cells were rinsed with PBS, and then supplied with the CS derivatives/DNA complexes in the same concentrations as in vitro transfection experiment. After treatment, CS derivatives/DNA complexes solutions were removed. Finally, the cells were incubated with 100 μL MTT containing medium (1 mg/mL) for 4 h. Then the medium was removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μL DMSO per well. Relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

2.10. Statistical analysis

Statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of N-(4-pyridinylmethyl) chitosans and methylated N-(4-pyridinylmethyl) chitosans

N-Pyridinylmethyl chitosans (Py-CS) were synthesized by reductive amination. The formation of Py-CS occurred via the corresponding Schiff base intermediates which were prior to borohydride reduction (Scheme 1). The pyridine carboxaldehydes at different mole ratios with respect to the glucosamine residues of CS were used. It was found that the *N*-substitutions (ES) were in the range of 13–62%, which was depended on molar ratio of aldehydes (Table 1). At the lower molar ratio of aldehyde to GlcN (1:1), ES was 40% while the higher ratio of aldehyde, the ES was 62%. The ^{1}H NMR spectrum of *N*-pyridinylmethyl chitosan was used to determine the ES by comparing the integral area of the H2 + H2′ + 1/3 *N*-acetyl proton signals with those of the aromatic protons of pyridinyl group (Crini et al., 1997). The methylation of CS and Py-CS were carried out using iodomethane under basic condition which yielded methylated chitosan and methylated Py-CS. The methylation occurred at primary amino groups of chitosan and N atom at *N*-pyridinylmethyl substituent. T e degree of quaternization (DQ) of chitosan and its methylated derivatives were determined by ^{1}H NMR spectroscopic method which was calculated from the relative peak area of *N,N,N*-trimethyl protons of GlcN to H1′ proton of the GlcN (Sieval et al., 1998). The DQ was depended on ES and the sodium hydroxide concentration. In our previously study, increasing the sodium hydroxide concentration led to the increase in DQ_{Ch} and *O*-methylation. Nevertheless, increasing ES did not increase DQ_{Ch}, due to lower numbers of unsubstituted GlcN units (Sajomsang et al., 2008). In addition, *O*-methylation at 3-hydroxyl and 6-hydroxyl positions was observed which found in the range of 5–10%.

The characterization of Py-CS and TM-Py-CS were confirmed by FT-IR and ^{1}H NMR spectra. The FT-IR spectra of chitosan exhibited the absorption bands at wave numbers 3430 cm^{-1} due to OH and NH_2 groups, 1648 and 1377 cm^{-1} corresponded to the C=O and C–O stretching of amide group, 1594 cm^{-1} due to N–H deformation of amino groups, 1155, 1081 and 1033 cm^{-1} corresponded to the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching (Brugnerotto et al., 2001). The FT-IR spectrum of Py-CS and TM-Py-CS were shown in Fig. 1. The absorption bands were similar to that of CS except the absorption band of C=C stretching corresponding to the aromatic group at wavenumbers 1607 and 1562 cm^{-1}, C–H stretching of quaternary ammonium group at wavenumber 1470 cm^{-1} and C–H deformation (out of plane) at wavenumber 846 cm^{-1}. The typical ^{1}H NMR spectrum of CS, Py-CS and TM-Py-CS was shown in Fig. 2. The CS exhibited the characteristic ^{1}H NMR pattern such as the singlet at δ 4.9 ppm due to H1, the multiplet at δ 4.4–3.5 ppm due to H3, H4, H5, H6, H6′ and two singlets at δ 3.2 and 2.1 ppm due to the H2 proton of the GlcN and *N*-acetyl protons of GlcNAc, respectively (Lavertu et al., 2003). Both spectra of Py-CS and TM-Py-CS exhibited a doublet of doublets at δ 8.6–8.0 ppm due to the protons of pyridine ring. For the additional signal, H1′ proton of the GlcN of TM-Py-CS appeared at δ 5.40 ppm and other additional signals at δ 4.2 ppm were due to the methyl protons at the N atom of pyridine ring, and the signals at δ 3.2, 2.7 and 2.3 ppm were assigned to *N,N,N*-trimethyl protons, *N,N*-dimethyl protons, and *N*-methyl protons of the GlcN of TM-Py-CS, respectively. In the present study, ES was determined from the relative peak area of pyridine protons to H2 proton of GlcN and *N*-acetyl protons of GlcNAc while DQ was calculated from the relative peak area of *N,N,N*-trimethyl protons of GlcN to H1′ proton of the GlcN.

Table 1
Methylation of chitosans and *N*-(4-pyridinylmethyl) chitosans

Samples	ES (%)	DQ_{Py} (%)	DQ_{CS} (%)	DQ_{Toral} (%)	$N(CH_3)_2$ (%)	$NHCH_3$ (%)	Recovery (%)
$TM_{43}CS$	–	–	43	43	48	ND	80
$TM_{65}CS$	–	–	65	65	23	ND	90
$TM_{52}Py_{13}CS$	13	13	39	52	20	7	76
$TM_{53}Py_{40}CS$	40	40	13	53	2	7	73
$TM_{69}Py_{62}CS$	62	62	7	69	10	13	70

ES is the extent of *N*-substitution; DQ_{Py} is degree of quaternization at N atom of pyridine ring; DQ_{CS} is degree of quaternization at GlcN of chitosan; $N(CH_3)_2$ is *N,N*-dimethylation at GlcN of chitosan; $NHCH_3$ is *N*-methylation at GlcN of chitosan; Recovery (%) is dried weight of methylated product (g)/dried weight of chitosan (g) or dried weight of *N*-(4-pyridinylmethyl) chitosan (g) × 100; ND is non-detectable.

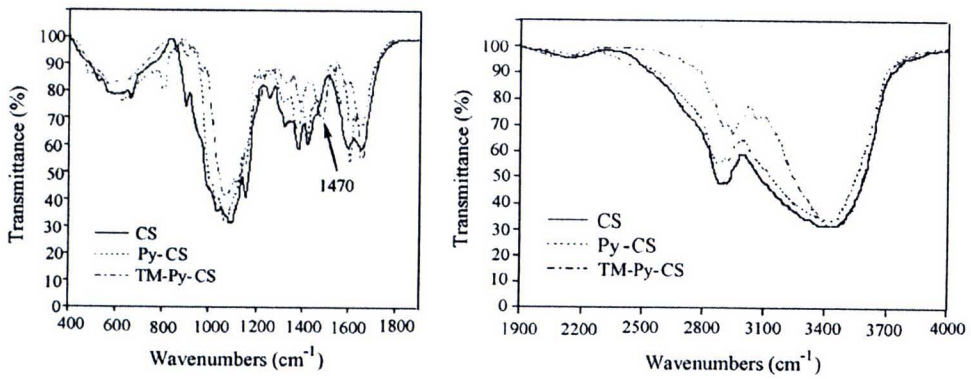


Fig. 1. FT-IR spectra of chitosan (CS), *N*-(4-pyridinylmethyl) chitosan (Py-CS) and methylated *N*-(4-pyridinylmethyl) chitosan (TM-Py-CS).

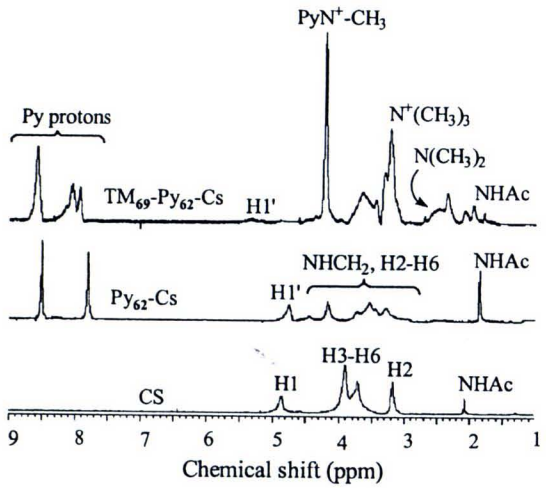


Fig. 2. ^{1}H NMR spectra of chitosan (CS), *N*-(4-pyridinylmethyl) chitosan (Py_{65}-CS) and methylated *N*-(4-pyridinylmethyl) chitosan ($TM_{69}Py_{62}CS$).

3.2. Characterization of CS derivatives/DNA complexes

In order to investigate the optimal conditions for the complex formation, it was necessary to evaluate the degree of binding between CS derivatives and DNA at different CS derivatives and DNA concentrations. The formation of complexes between CS derivatives and pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. Fig. 3a shows the naked DNA (Lane 2) and $TM_{52}Py_{13}CS$/DNA complexes at weight ratios of 2, 4, 6, 8, 12 and 16 (Lane 3–8). Compared with the naked DNA, DNA which formed complexes was retarded in migration. For $TM_{52}Py_{13}CS$, the entire DNA appeared to be retained in the loading well, illustrating that DNA had completely formed the complexes with $TM_{52}Py_{13}CS$ at weight ratio higher than 8. In a case of $TM_{53}Py_{40}CS$, complexes were completely formed at the weight ratio above 1.8 (Fig. 3b), whereas for $TM_{69}Py_{62}CS$ the weight ratio was above 1.1 (Fig. 3c). These results clearly showed that the complete formation of complex occurred at lower weight ratio if the CS with higher degree of *N*-4-pyridinylmethyl substitution were used. Since the structure of DNA comprised of aromatic bases, these charge-neutralized moieties probably interact via hydrophobic force with *N*-4-pyridinylmethyl groups of CS derivatives and accountable for the enhanced DNA condensation. The hydrophobic moiety-dependent gene condensation capacity phenomenon is in close agreement with the previously reported results (Chae et al., 2005).

Particle size and the zeta potential were plotted against weight ratios of CS derivatives/DNA complexes formulated (Fig. 4). The particle size of the complete CS derivatives/DNA complexes were in the range of 200 to 400 nm. Initially, negative values of zeta potentials were observed at low weight ratio. However, when the complete complexes were formed ($TM_{52}Py_{13}CS$ above weight ratio of 8; Fig. 4a, $TM_{53}Py_{40}CS$ above weight ratio of 1.4; Fig. 4b and $TM_{69}Py_{62}CS$ above weight ratio of 1.1; Fig. 4c), the zeta potential values became approximately neutral. The zeta potential of

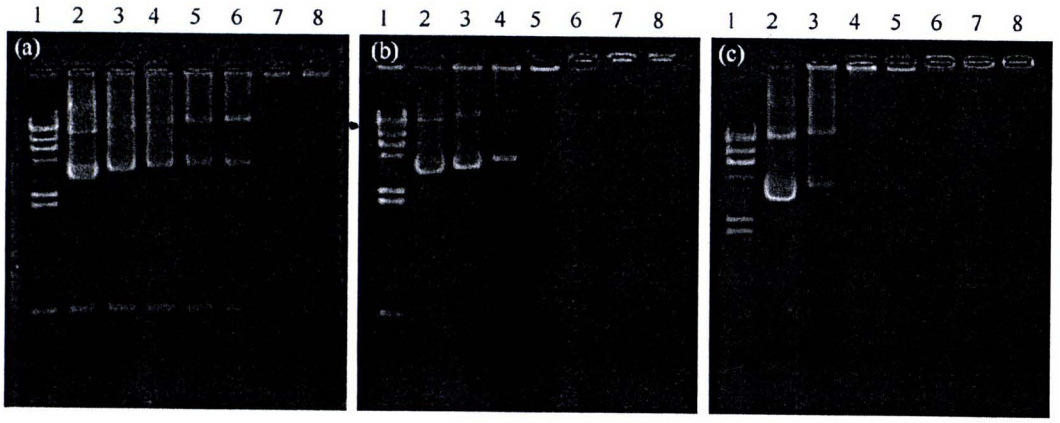


Fig. 3. Gel retarding analysis of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$ and (c) $TM_{69}Py_{62}CS$. Lane 1, DNA marker; Lane 2, pEGFP-C2 plasmid; Lanes 3–8, $TM_{52}Py_{13}CS$/DNA complexes (weight ratios of 2, 4, 6, 8, 12 and 16) $TM_{53}Py_{40}CS$/DNA complexes (weight ratios of 1.4, 1.8, 2.1, 4, 6 and 8) and $TM_{69}Py_{62}CS$/DNA complexes (weight ratios of 1.1, 1.6, 2, 4, 6 and 8).

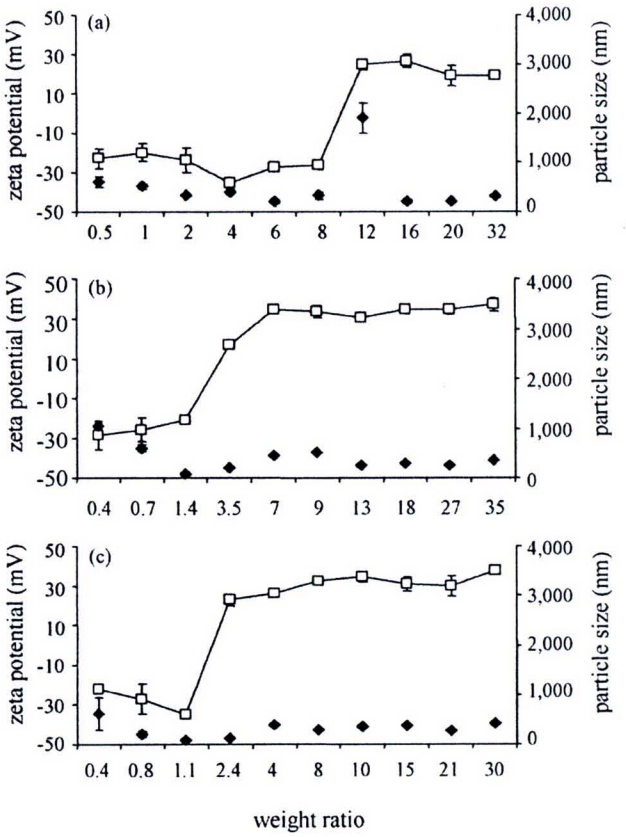


Fig. 4. Zeta potential (□) and particle size (♦) at varying weight ratios of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$ and (c) $TM_{69}Py_{62}CS$. Each value represents the mean ± S.D. of three measurements.

the complexes was found to be more positive with the increase in weight ratios of CS derivatives due to their higher density of quaternization in the CS backbone.

3.3. In vitro transfection

The achievement of high gene transfection efficiency is a final goal for the development of novel gene carriers. To investigate the CS derivative mediated gene transfection efficiencies, in vitro gene transfection assay was performed with human hepatoma cell lines (Huh 7 cells) using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). CS derivatives/DNA complexes were formulated with various weight ratios in order to investigate the optimal conditions for gene transfection. Polyethylenimine (PEI, 25 kDa) complexed with DNA at the weight ratio of 1 was used as a positive control. In all studies, there were no transfection in control (cells without complexes) and naked DNA. As shown in Fig. 5, the gene transfection efficiencies at pH 7.4 were significantly influenced by weight ratios. By increasing the weight ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. Among the carriers, $TM_{69}Py_{62}CS$ showed the highest transfection efficiency (Fig. 5c). Its highest transfection efficiency at weight ratio of 4 was 33 times higher in gene transfection than that of TM_{65}-CS (Fig. 5e). These results revealed that not only the trimethyl quaternization but also the *N*-4-pyridinylmethyl substitution affected the gene transfection efficiency. Increasing the *N*-4-pyridinylmethyl substitution increased the gene transfection efficiency (Fig. 5a–c). In comparison, the highest transfection efficiency of $TM_{69}Py_{62}CS$ (weight ratio of 4), $TM_{53}Py_{40}CS$ (weight ratio of 4) and $TM_{52}Py_{13}CS$ (weight ratio of 16) were 1137, 248 and 83 cells/cm^2, respectively. Partially quaternized polymers have been previously referred as promising transfection agents. Cationic polymers containing quaternary charged trimethylamino ethylmethacrylate (TMAEM) copolymerized with hydrophilic-(2-hydroxypropyl) methacrylates (HPMA) were found to condense DNA and transfect 293 cells (Curti et al., 2003). Trimethylated chitosan oligomers are promising agents for DNA condensation and promote the transfection efficiency on COS-1, Caco-2 cells (Thanou et al., 2002), COS-7 and MCF-7 cells (Kean et al., 2005). This permanent positive charge of the trimethylated chitosan is a key factor for the condensation and protection of DNA. The introduction of *N*-4-pyridinylmethyl group into the CS polymer backbone enhances the hydrophobicity which improves the hydrophobic interaction between polymer and DNA and DNA condensation. In addition, it improves hydrophobic interaction with cell membrane (Chae et al., 2005; Doody et al., 2006). These help the water-soluble CS to be an efficient vector. As reported previously, hydrophobically modified cationic polymers or cationic lipids have shown high gene transfection capability as an optimal substitution by increasing cell membrane/carrier interactions or destabilization of the cell membranes (Tian et al., 2007). In our previous study, the introduction of 4-*N*,*N*-dimethylaminobenzyl group into the CS polymer backbone enhanced the hydrophobicity also increased the transfection efficiency (Rojanarata et al., 2008). Moreover, *N*-4-pyridinylmethyl moieties on the CS backbone showed more efficient gene transfection than 4-*N*,*N*-dimethylaminobenzyl moieties. Our results reveal that the chemical structures of hydrophobic moieties play an important role for gene transfection. Although the exact mechanism of $TM_{69}Py_{62}CS$ mediated efficient gene delivery remain to be further studied, our results showed that $TM_{69}Py_{62}CS$ could be potential candidate for non-viral gene carriers.

3.4. Effect of CS derivatives/DNA complexes on cell viability

One of the major requirements for cationic polymer vectors for gene delivery is low cytotoxicity. It has been reported that CS and CS derivatives were less toxic than other cationic polymers such as poly-lysine and polyethyleneimine in vitro and in vivo (Thanou et al., 2002). Various chitosans and chitosan derivatives have been reported for gene delivery. However, the toxicity of those chitosans was different depending on the type of cells and derivatives studied. Therefore, the cytotoxicity study of the CS derivatives/DNA complex was performed in Huh 7 cells. Fig. 6 shows the effect of $TM_{52}Py_{13}CS$/DNA (Fig. 6a), $TM_{53}Py_{40}CS$/DNA (Fig. 6b), $TM_{69}Py_{62}CS$/DNA (Fig. 6c), $TM_{43}CS$/DNA (Fig. 6d), and $TM_{65}CS$/DNA complexes (Fig. 6e) on cell viability. When Huh 7 cells were incubated with 1 μg of naked DNA, cell viability remained almost the same as that seen in control non-transfected cells (data not shown). The significant decrease in cell viability was observed when Huh 7 cells were incubated with increasing weight ratios of $TM_{43}CS$/DNA and $TM_{65}CS$/DNA complexes, whereas a slight decrease was found in $TM_{52}Py_{13}CS$/DNA, $TM_{53}Py_{40}CS$/DNA, and $TM_{69}Py_{62}CS$/DNA complexes. However, the viability was over 80% at the weight ratio of 4 where the highest transfection efficiency was obtained. Therefore, from this study, the $TM_{69}Py_{62}CS$ is clearly proved to be safe.

The transfection results of $TM_{69}Py_{62}CS$/DNA complexes (Fig. 5c) showed that when the weight ratio was increased above 4, transfection efficiency significantly decreased, whereas cytotoxicity slightly decreased, except for that at weight ratio of 48. These results revealed that cytotoxicity hardly involved in the reduced level of transgene expression by $TM_{69}Py_{62}CS$/DNA complexes prepared at higher weight ratios. Therefore, other factors might be involved in the reduced transgene expression. The decrease in transfection efficiency of the complexes might be explained by large

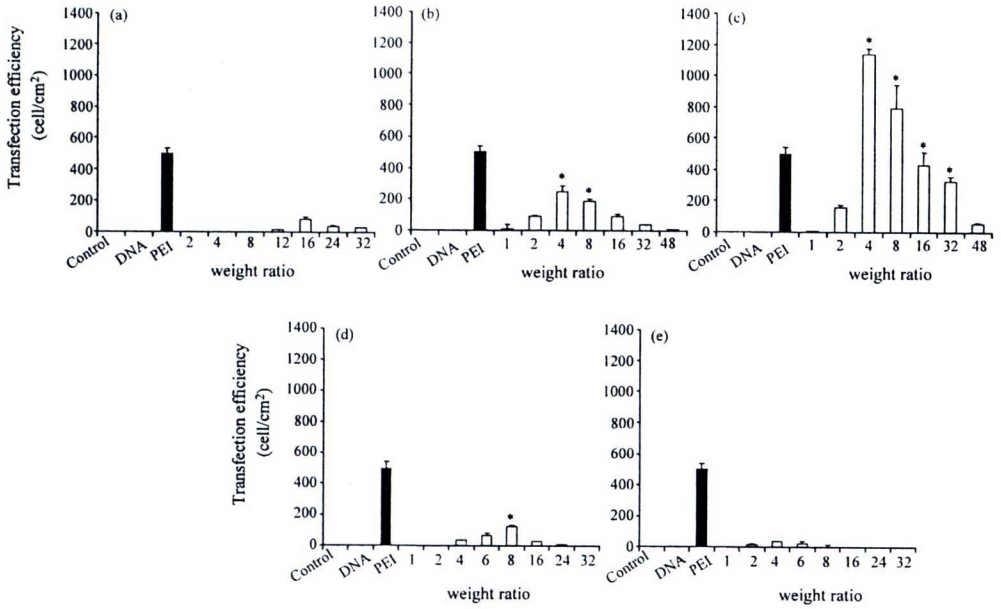


Fig. 5. Transfection efficiencies of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$, (c) $TM_{69}Py_{62}CS$, (d) TM_{43}-CS and (e) TM_{65}-CS in Huh 7 cells. Each value represents the mean ± S.D. of nine wells. Difference values * were statistically significant ($p < 0.05$).

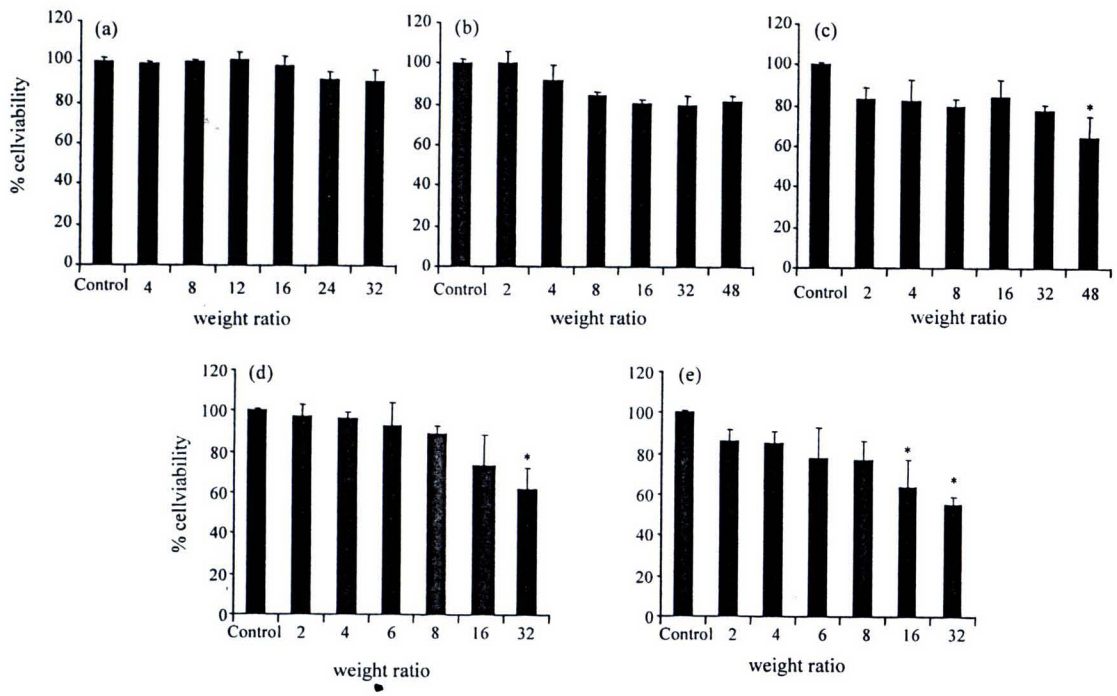


Fig. 6. Cell viability of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$, (c) $TM_{69}Py_{62}CS$, (d) TM_{43}-CS and (e) TM_{65}-CS in Huh 7 cells. Each value represents the mean ± S.D. of nine wells. Difference values * were statistically significant ($p < 0.05$).

amount of polymer which inhibited the cellular internalization of complexes (Furuhata et al., 2008) or the release of DNA from the complexes into cytoplasm (Wang et al., 2002).

4. Conclusion

In this study, the novel water-soluble chitosan derivatives ($TM_{69}Py_{62}CS$) were successfully synthesized for improving transfection efficiency by chemically modified with hydrophobic moiety of *N*-4-pyridinylmethyl and increasing solubility by *N*-quaternization. This study suggests that $TM_{69}Py_{62}CS$ is safe and exhibits significantly improved gene delivery potential in vitro.

Acknowledgments

The authors would like to acknowledge the financial support from the Research, Development and Engineering (RD&E) Fund through National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Thailand

(Project No. B21-CR0169-10RDCR01) and Thailand Research Funds (Project No. DBG5180005).

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Carbohydrate Polymers 75 (2009) 143–149

Contents lists available at ScienceDirect

Carbohydrate Polymers

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Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan as effective gene carriers: Effect of degree of substitution

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ARTICLE INFO

Article history:
Received 11 March 2008
Received in revised form 7 July 2008
Accepted 8 July 2008
Available online 13 July 2008

Keywords:
Methylated *N*-(4-*N N*-dimethylaminobenzyl) chitosan
Gene delivery
Huh7 cells
Transfection efficiency
DNA vector

ABSTRACT

The objective of this study was to investigate the transfection efficiency of quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan; TM-Bz-CS, using the plasmid DNA encoding green fluorescent protein (pEGFP-C2) on human hepatoma cell lines (Huh7 cells). The factors affecting the transfection efficiency e.g. degree of quaternization (DQ), the degree of dimethylaminobenzyl substitution (DS) and polymer/DNA weight ratio, have been evaluated. The results revealed that all TM-Bz-CS derivatives were able to condense with DNA. Illustrated by agarose gel electrophoresis, complete complexes of TM_{57}-Bz_{42}-CS/DNA were formed at weight ratio of above 0.5, whereas those of TM_{47}-Bz_{42}-CS/DNA and TM_{57}- Bz_{17}-CS/DNA were above 1. The rank of transfection efficiency of the chitosan derivatives were TM_{57}-Bz_{42}-CS > TM_{47}-Bz_{42}-CS > TM_{57}-Bz_{18}-CS. The pH of culture medium did not affect the transfection efficiency of TM_{57}-Bz_{42}-CS/DNA complex, whereas it affected the transfection efficiency of chitosan/DNA complex. The results indicated that the improved gene transfection was due to the hydrophobic group (*N*,*N*-dimethylaminobenzyl) substitution on chitosan which promoted the interaction and condensation with DNA as well as *N*-quaternization which increased chitosan water solubility and enhance gene expression. For cytotoxicity studies, TM-Bz-CS was safe at the concentration of the highest transfection. In conclusion, this novel chitosan derivative, TM_{57}-Bz_{42}-CS showed elevated potential as gene carrier by efficient DNA condensation and mediated highest level of gene transfection with negligible cytotoxicity in Huh7 cells.

1. Introduction

As a non-viral vector for gene delivery, chitosan has several advantages over viral vectors since it does not cause virally-induced inflammatory responses, immunological reactions and oncogenic effects (Simon et al., 1993). In addition, chitosan is biocompatible, biodegradable and non-toxic; therefore, it has been proposed as a safer alternative to other non-viral vectors such as cationic lipids and cationic polymers (Kumar et al., 2003; Lee et al., 2001; Thanou, Florea, Geldof, Junginger, & Borchard, 2002; Weecharangsan, Opanasopit, Ngawhirunpat, Rojanarata, & Apirakaramwong, 2006). At acidic pH, below pK_a, the primary amines in the chitosan backbone become positively charged (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). These protonated amines enable chitosan to bind to negatively charged DNA and condense it into particles. Chitosan has shown promise to protect DNA from DNase I and II degradation (Huang, Fong, Khorc, & Lim, 2005; Richardson, Kolbe, & Duncan, 1999) and transfect into different cell types (Ishii, Okahata, & Sato, 2001; Maclaughlin et al., 1998). Formulation parameters such as molecular weight (MW), degree of deacetylation (DD), N/P ratio (molar ratio of chitosan nitrogen (N) per DNA phosphate (P)), and pH of transfection medium were found to affect the transfection efficiency of chitosan/DNA complexes (Ishii et al., 2001; Kiang, Wen, Lim, & Leong, 2004; Lavertu, Méthot, Tran-Khanh, & Buschmann, 2006; Romóren, Pedersen, Smistad, Evensen, & Thu, 2003; Sato, Ishii, & Okahata, 2001; Weecharangsan et al., 2008).

The main drawback of chitosan would be the poor water solubility at physiological pH and low transfection efficiency. But several chitosan derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics (Rinaudo, 2006). Modified chitosan such as glycol chitosan or PEGylated chitosan (Yoo, Lee, Chung, Kwon, & Jeong, 2005; Zhang et al., 2007), low molecular weight soluble chitosan (Lee et al., 2001), quaternized CS (Thanou et al., 2002), and polyethylenglycol-graft-trimethyl chitosan (Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008) could be possible ways to circumvent the solubility issues. To improve gene transfection, chemically modified chitosan, such as quaternized chitosan

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doi:10.1016/j.carbpol.2008.07.012

(Thanou et al., 2002), urocanic acid-modified chitosan (Kim, Ihm, Choi, Nah, & Cho, 2003), galactosylated chitosan (Gao et al., 2005), deoxycholic acid chitosan oligosaccharide nanoparticle (Chae, Son, Lee, Jang, & Nah, 2005) and thiolated chitosan (Lee et al., 2007) were proposed.

Although many researches synthesized chitosan derivatives as alternative for gene carrier, a few were successful in increased transfection efficiency. Recently, our research groups have successfully synthesized the novel water soluble chitosan derivatives namely methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan. This chitosan derivatives showed elevated potential as gene carrier by efficient DNA condensation and mediated higher level of gene transfection with negligible cytotoxicity in Huh7 cells (Rojanarata et al., 2008). In this study, methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan with various substitutions was synthesized. A number of variables that influenced transfection efficiency such as the degree of dimethylaminobenzyl substitution (DS), degree of quaternization (DQ) and weight ratio were determined. The physical properties of the complexes were investigated. Their transfection efficiencies and cytotoxicity in human hepatocellular carcinoma cells (Huh7 cells) were evaluated.

2. Experimental

2.1. Materials

Chitosan was purchased from Seafresh Chitosan Laboratory, Thailand, with MW of 267 kDa and 94% degree of deacetylation. Sodium cyanoborohydride and polyethylenimine (PEI), MW 25 kDa, were purchased from Aldrich, Germany. 4-*N*,*N*-Dimethylaminobenzaldehyde, iodomethane, sodium iodide and 1-methyl-2-pyrrolidone were purchased from Fluka, Germany. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM), Trypsin–EDTA, penicillin–streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen, USA. The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, USA. The λ*Hin*dIII was obtained from Promega, USA. Huh7 (Human hepatocellular carcinoma) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

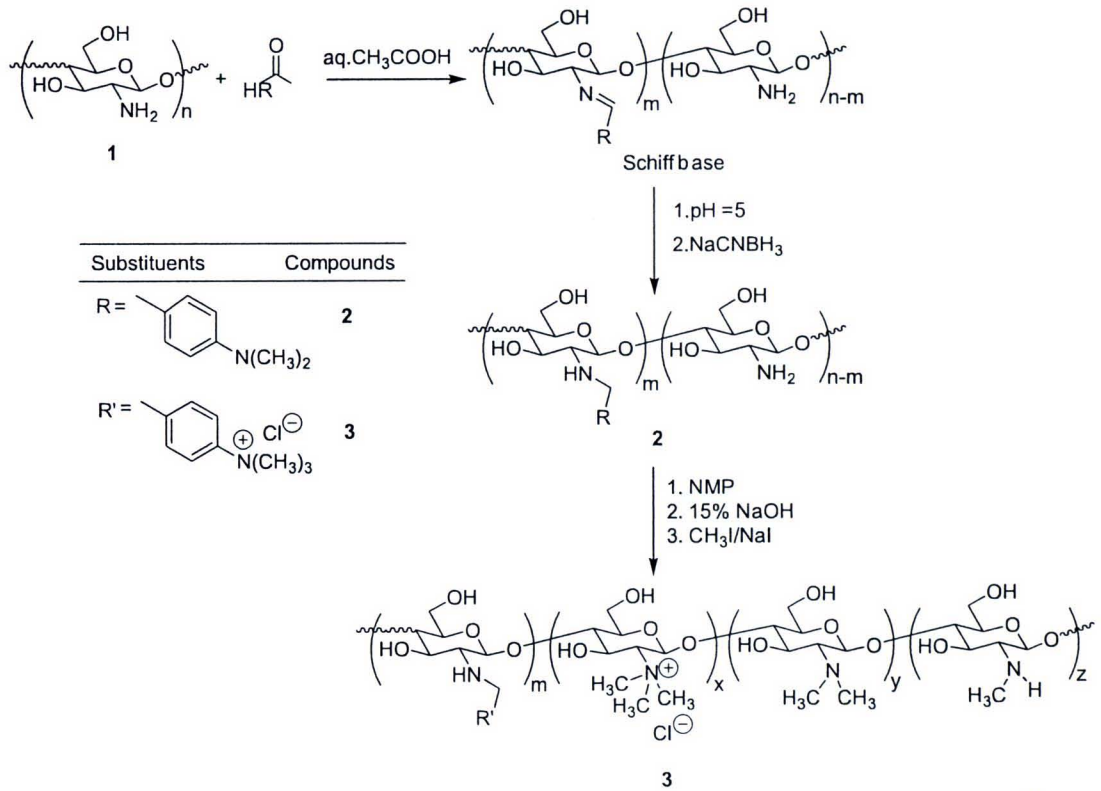
2.2. Synthesis of methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS)

2.2.1. Synthesis of N-(4-N,N-dimethylaminobenzyl) chitosan (DM-Bz-CS)

DM-Bz-CS **2** was prepared as previously reported (Sajomsang, Tantayanon, Tangpasuthadol, & William, 2008). Briefly, 1.00 g of chitosan was dissolved in 70 mL of 1% acetic acid solution. The solution was diluted with ethanol (70 mL). 4-*N*,*N*-Dimethylamino benzaldehyde (3.0 mmol) was then added and the solution was stirred at room temperature for 1 h. The pH of the solution was adjusted to 5 with 1 N NaOH. Subsequently, 1.54 g of $NaCNBH_3$ was added and stirred at room temperature for 24 h, followed by the pH adjustment to 7 with 15% (w/v) NaOH. The reaction mixture was then dialyzed in distilled water and freeze-dried to give powder **2** (Scheme 1).

2.2.2. Synthesis of TM-Bz-CS

0.50 g of compound **2** was dispersed in 25 ml of *N*-methyl pyrrolidone (NMP) for 12 h at room temperature. Then 1.5 g of sodium



Scheme 1. Synthesis of methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan.

iodide 15% and 5% w/v NaOH (3.0 ml) were added and stirred at 50 °C for 15 min. Subsequently, 1 mL of methyl iodide was added in three portions at 4 h intervals and stirred for 12 h at 50 °C. The reaction mixture appeared yellow and clear. The obtained compounds were purified by precipitation in 300 mL of acetone. The precipitate was dissolved in 15% (w/v) NaCl solution in order to replace the iodide ion by chloride ion. The suspension was dialyzed with deionized water for 3 days to remove inorganic materials. The dialyzed solution was then concentrated under vacuum on a rotary evaporator and then precipitated in acetone (100 mL). The pure compound (TM-Bz-CS **3**) was collected and dried overnight at room temperature under a stream of nitrogen (Scheme 1).

2.3. Characterizations

FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared (FT-IR) spectrometer. All samples were prepared as potassium bromide pellets. The ^{1}H, ^{13}C NMR spectra were measured on a Mercury Varian 300 MHz spectrometer. All measurements were performed at 300 K, using pulse accumulating of 64 scans and the LB parameter of 0.30 Hz. One percent (v/v) D_2O/CF_3COOD and D_2O was a solvent for 10 mg chitosan and its derivatives, respectively.

2.4. Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA). DNA concentration was quantified by the measurement of UV absorbance at 260 nm using a GeneRay UV Photometer (Biometra). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate–EDTA (TAE) buffer, pH 8.0, using λDNA/*Hin*dIII as a DNA marker.

2.5. Preparation and characterization of chitosan derivatives /DNA complexes

The chitosan derivatives/DNA complexes were prepared at various weight ratios by adding the DNA solution to the chitosan derivative solution. The mixture was gently mixed using pipette for 3–5 s to initiate complex formation and left for 15 min at room temperature. The complex formation was confirmed by electrophoresis. Agarose gels were prepared with 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/ml). The electrophoresis was carried out for 60 min at 100 V. The volume of the sample loaded in the well was 15 μl of chitosan derivatives/DNA complex containing 1 μg of DNA.

2.6. Size and zeta potential measurements

The particle size and surface charge of chitosan derivatives/DNA complexes were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior to use. All samples were measured in triplicate.

2.7. In vitro transfection chitosan derivatives/DNA complexes in Hun7cells

Huh7 cells were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 ml of growth medium (DMEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air, 37 °C) for 24 h. Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). The cells were incubated with 0.5 ml of the chitosan derivatives/DNA complexes at various weight ratios containing 1 μg of pDNA for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes were used as controls. After transfection, the cells were washed twice with PBS and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

2.8. Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. Huh7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μl of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to transfection, the medium was removed and the cells were rinsed with PBS. The cells were then treated with the chitosan derivatives/DNA complexes in the same concentrations as in vitro transfection experiment and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells were used as controls incubated for the same duration of time. After treatment, chitosan derivatives/DNA complexes solutions were removed. Finally, the cells were incubated with 100 μl MTT containing medium (1 mg/ml) for 4 h. Then the medium was removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μl DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

2.9. Statistical analysis

Statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < .05$.

3. Results and discussion

3.1. Synthesis and characterization of chitosan derivatives

As previously reported, DM-Bz-CS **2** was synthesized by reductive amination of the corresponding Schiff base intermediates (Scheme 1) (Sajomsang et al., 2008). The degree of *N*-substitution (DS), determined by ^{1}H NMR varied due to the different ratio of aldehydes to D-glucosamine (GlcN) of chitosan. The FT-IR spectra of DM-Bz-CS were similar to that of chitosan except that the additional absorption bands at wave numbers 1605, 1526, and 811 cm^{-1} were observed. These bands were assigned to C=C stretching and C—H deformation (out of plane) of the aromatic group. These results are in agreement with those already published for benzylated chitosans (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982). The ^{1}H NMR spectrum of DM-Bz-CS exhibited the broad singlet, δ 7.5 ppm, in the aromatic region and another singlet at δ 3.1 ppm assigned to *N,N*-dimethyl protons (Fig. 1). The ^{1}H NMR spectra of TM-Bz-CS were similar to that of the corresponding TM-CS except the additional signals at δ 3.5 ppm which was of *N,N,N*-trimethyl protons on benzyl substituent.

In this study, the quaternization of DM-Bz-CS **2** was based on a nucleophilic substitution of the primary amino group on the C-2 position of chitosan, using the procedures which was slightly modified from the method previously described (Curti, de Britto, & Campana-Filho, 2003). The quaternization of DM-Bz-CS **2**, with methyl iodide yielded TM-Bz-CS **3**, could occur at both the

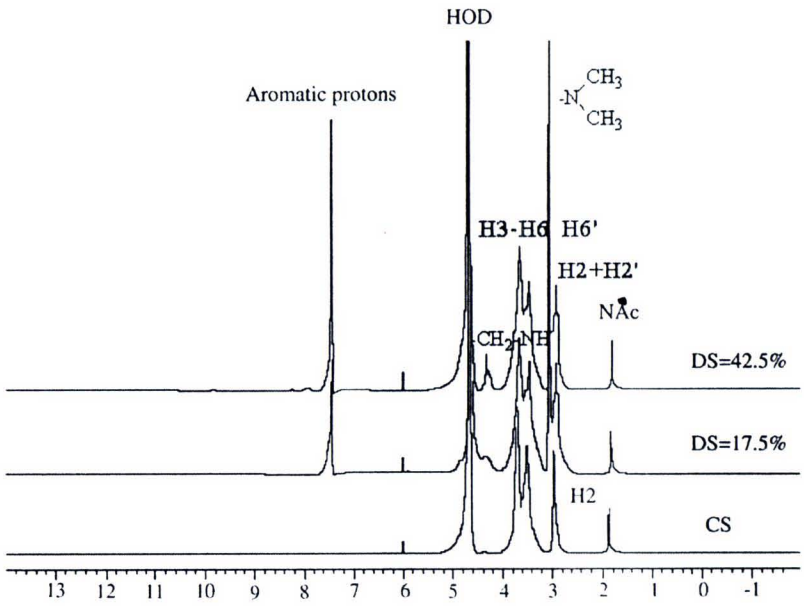


Fig. 1. ^{1}H NMR spectra of chitosan (CS) and *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (DM-Bz-CS).

aromatic substituent and primary amino group of GlcN of chitosan. The results clearly demonstrated that *N*,*N*-dimethylamino groups of DM-Bz-CS **2** were more reactive than the primary amino groups of chitosan which were completely quaternized giving DQ_{Ar} values equal to the corresponding DSs (Table 1). Besides *N*,*N*,*N*-trimethylation, *N*,*N*-dimethylation and *N*-monomethylation at the primary amino group of GlcN of chitosan were also observed.

3.2. Characterization of chitosan derivatives/DNA complexes

In order to determine the optimal complexation conditions, it was necessary to evaluate the formation of complexes between chitosan derivatives and DNA at different chitosan derivatives/DNA weight ratios. The formation of complexes was visualized by agarose gel electrophoresis. Fig. 2a shows the naked DNA (Lane 2) and $TM_{57}Bz_{18}CS$ /DNA complexes at weight ratios of 0.5, 1, 2, 4, 6 and 8 (Lanes 3–8). The naked DNA lane showed the DNA band, whereas complexed DNA was completely retained within the gel-loading well for $TM_{57}Bz_{18}CS$ at weight ratio above 1, illustrating that complete $TM_{57}Bz_{18}CS$/DNA complexes were formed. Fig. 2b shows that complexes were completely formed at weight ratio above 1for TM_{47}-Bz_{42}-CS, whereas complete complexes for TM_{57}-Bz_{42}-CS were formed at weight ratio above 0.5 (Fig. 2c). These results revealed that the hydrophobic interactions between dimethylaminobenzyl moieties of chitosan derivatives and charge-neutralized DNA segments were possibly accountable for the enhanced gene condensation.

Particle size and the zeta potential were plotted against weight ratios of chitosan derivatives/DNA complexes formulated (Fig. 3). The particle size of the TM_{57}-Bz_{42}-CS/DNA complexes increased with the increasing weight ratio from 0.5 to 1 and decreased to constant value in the range of 200 to 300 nm after a weight ratio of 1 (Fig. 3c). At the weight ratio of 1, TM_{57}-Bz_{42}-CS/DNA complexes had the largest particle size. An initial negative value of the zeta potential was observed at a low weight ratio of 1. At the weight ratio between 0.5 and 1, the zeta potential was approximately neutral. On the other hand, the particle size of the TM_{57}-Bz_{18}-CS/DNA and TM_{47}-Bz_{42}-CS/DNA complexes increased with the increasing weight ratio from 1 to 2 and decreased to constant value in the range of 176–300 nm after a weight ratio of 2 (Fig. 3a and b). The zeta potential of the complexes was found to increase with the increase in weight ratios of chitosan derivatives due to their higher density of protonated amines in the chitosan backbone. These results clearly showed that the complete formation of complex occurred at lower weight ratio if the chitosan with higher degree of dimethylaminobenzyl substitution were used. The heterocyclic bases present in DNA probably associated hydrophobically with dimethylaminobenzyl groups and promoted enhanced interaction of DNA with the modified chitosan in agreement with previous results (Chae et al., 2005).

3.3. In vitro transfection

The achievement of high gene transfection efficiency is a final goal for the development of novel gene carriers. To investigate

Table 1
Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan

Chitosan derivatives	DS (%)	DQ (%)			DMCS (%)	MCS (%)	Yield (%)
		DQ_{Ar} (%)	DQ_{CS} (%)	DQ_T (%)			
TM_{57}-Bz_{42}-CS	42.5	42.5	14.4	56.9	Trace	11.7	84.0
TM_{47}-Bz_{42}-CS	42.5	42.5	4.4	46.9	1.7	6.7	78.0
TM_{57}-Bz_{18}-CS	17.5	17.5	40.0	57.5	16.7	16.7	68.0

DS is the degree of dimethylaminobenzylation; DQ_{Ar} is the degree of quaternization at the aromatic amines; DQ_{CS} is the degree of quaternization of the primary amine of chitosan; DMCS is the degree of *N*,*N*-dimethylation of the primary amine of chitosan; MCS is *N*-monomethylation at the primary amine of chitosan; Yield (%) is (weight of product (g)/weight of initial reactant (g)) × 100, at the end of the preparation.

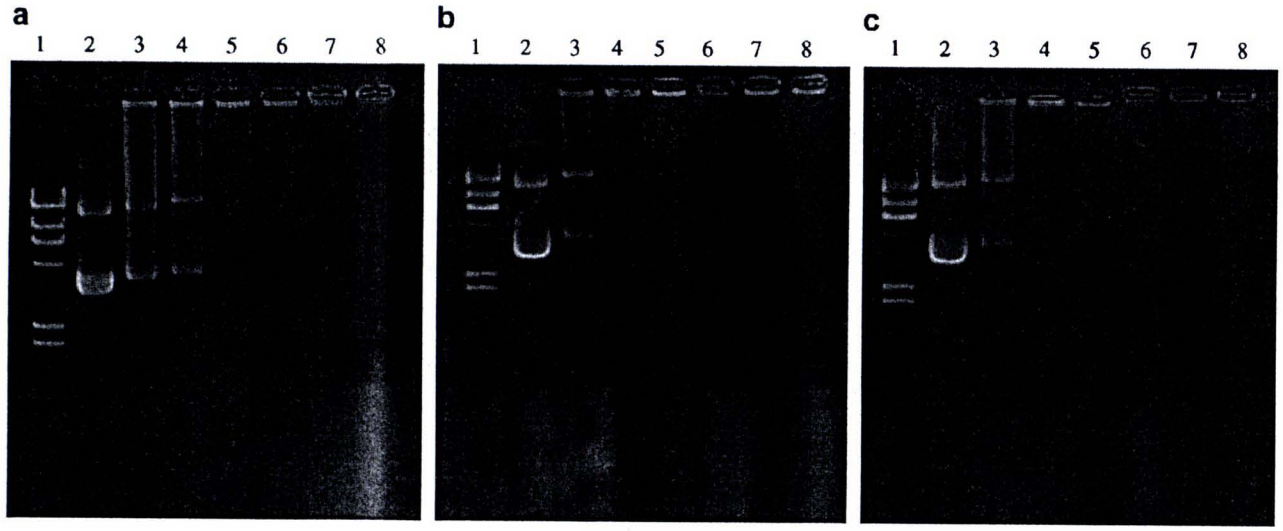


Fig. 2. Gel retarding analysis of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS. Lane 1, DNA marker and Lane 2 pEGFP-C2 plasmid; lanes 3-8, TM-Bz-CS/DNA complexes at weight ratios of 0.5, 1, 2, 4, 6, and 8, respectively.

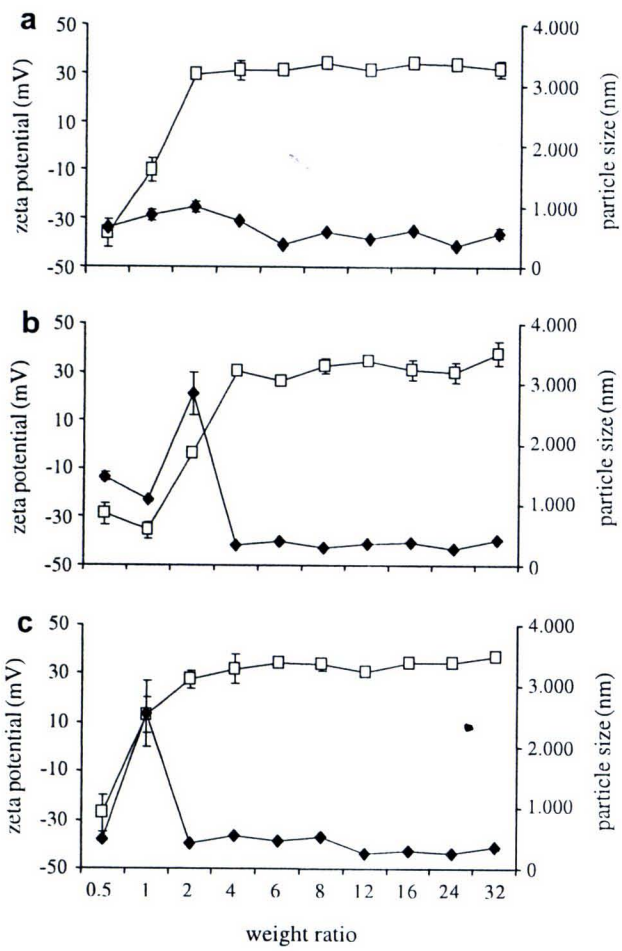


Fig. 3. Zeta potential (□) and particle size (♦) at varying weight ratios of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS. Each value represents the mean ± SD of three measurements.

the chitosan derivative mediated gene transfection efficiencies, in vitro gene transfection assay was performed with human hepatoma cell lines (Huh7 cells) using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). Chitosan derivatives/DNA complexes were formulated with various weight ratios (0.5, 1, 2, 4, 6, 8, 12, 16, 24 and 32) in order to investigate the optimal conditions for gene transfection. Polyethylenimine (PEI, 25 kDa) complexed with DNA at the weight ratio of 1 was used as a positive control. In all studies, there were no transfection in control (cells without complexes) and naked DNA. As shown in Fig. 4 (at pH 7.4), the gene transfection efficiencies were significantly influenced by the weight ratios, the degree of dimethylaminobenzyl substitution (DS) and degree of quaternization (DQ). By increasing the weight ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. Among chitosan derivatives, TM_{57}-Bz_{42}-CS showed the highest transfection efficiency (Fig. 4c). Its highest transfection efficiency at weight ratio of 16, was 8.3 and 12 times higher in gene transfection than that of the highest transfection efficiency of TM_{43}-CS at weight ratio of 8 (Rojanarata et al., 2008) and chitosan at weight ratio of 2 (Rojanarata et al., 2008), respectively. These results revealed that not only the trimethyl groups but also the hydrophobic groups (trimethylaminobenzyl moieties) affected the gene transfection efficiency. Increasing the hydrophobic groups (from Bz-18 to Bz-42) increased the gene transfection efficiency (Bz-18; Fig. 4a and Bz-42; Fig. 4b and c). The highest transfection efficiency of TM_{57}-Bz_{42}-CS was 580 ± 56 cells/cm^2 at weight ratio of 16, whereas the highest transfection efficiency of TM_{47}-Bz_{42}-CS and TM_{57}-Bz_{18}-CS were 435 ± 40 cells/cm^2 at weight ratio of 8 and 46 ± 7 cells/cm^2 at weight ratio of 12, respectively.

Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 (Weecharangsan et al., 2008). The effect of pH on transfection efficiencies of TM_{57}-Bz_{42}-CS/DNA and chitosan/DNA complexes were shown in Fig. 5. TM_{57}-Bz_{42}-CS mediated gene delivery (weight ratio of 16) about 2.5 times and 12 times enhanced gene transfection was observed than that of chitosan (weight ratio of 2) at the medium pH values of 6.5 and 7.4, respectively. pH dramatically affected only chitosan with five times decreasing the transfection efficiency by increasing pH values from 6.5 to 7.4, while TM_{57}-Bz_{42}-CS was not significantly influenced by pH.

Trimethylated chitosan (Mao et al., 2007) and trimethylated chitosan oligomers are promising agents for DNA condensation and promote the transfection efficiency on COS-1, Caco-2 cells

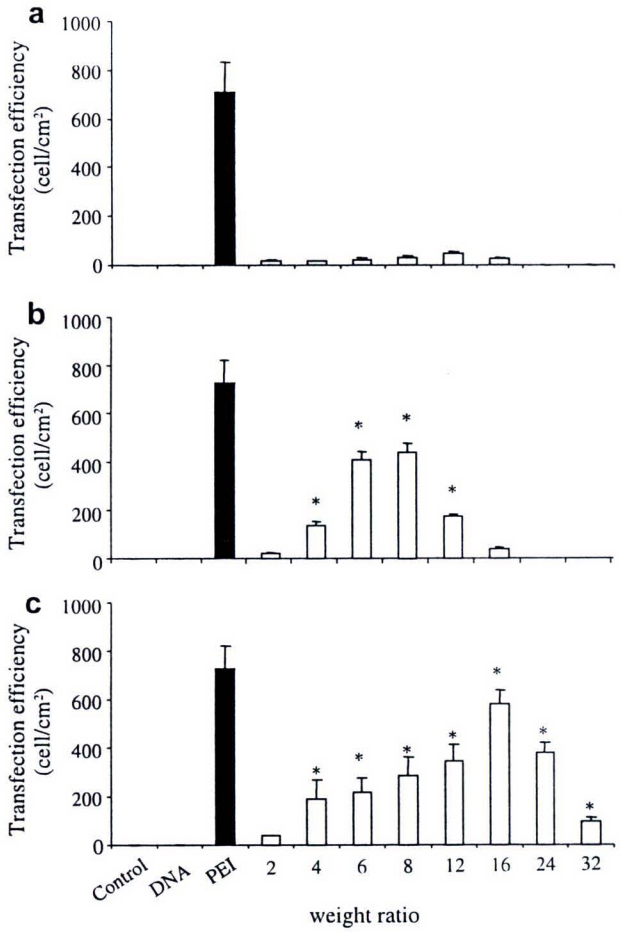


Fig. 4. Transfection efficiencies of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS in Huh7 cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < .05$).

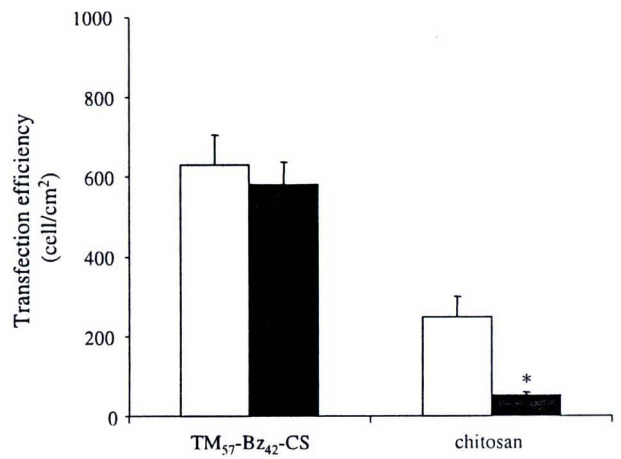


Fig. 5. Effect of pH medium at: (white bar) pH 6.5 and (black bar) pH 7.4; on transfection efficiencies of TM_{57}-Bz_{42}-CS/DNA complexes and chitosan/DNA complexes at the weight ration of 16 and 2, respectively, in Huh7 cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < .05$).

(Thanou et al., 2002), COS-7 and MCF-7 cells (Kean, Roth, & Thanou, 2005). This permanent positive charge of the trimethylated

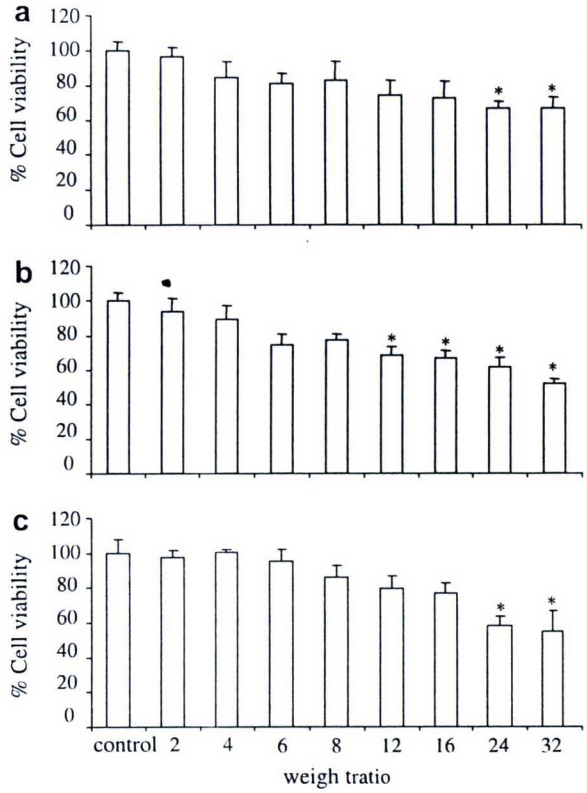


Fig. 6. Cell viability of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS in Huh7 cells. Each value represents the mean ± SD of six wells. Difference values * were statistically significant ($p < .05$).

chitosan is a key factor for the condensation and protection of DNA. The introduction of trimethylaminobenzyl group into the chitosan polymer backbone enhances the hydrophobicity which improves the hydrophobic interaction between polymer and DNA and DNA condensation. In addition, it improves hydrophobic interaction with cell membrane (Chae et al., 2005; Doody, Korley, Dang, Zawaneh, & Putnam, 2006). These help the water-soluble chitosan to be an efficient vector. As reported previously, hydrophobically modified cationic polymers or cationic lipids have shown high gene transfection capability as an optimal substitution by increasing cell membrane/carrier interactions or destabilization of the cell membranes (Tian et al., 2007). Although the exact mechanism of TM-Bz-CS mediated efficient gene delivery remained to be further studied, our results showed that could be suitable for non-viral gene carriers.

3.4. Effect of chitosan derivatives/DNA complexes on cell viability

One of the major requirements for cationic polymer vectors for gene delivery is low cytotoxicity. It has been reported that chitosan and chitosan derivatives were less toxic than other cationic polymers such as poly-lysine and polyethyleneimine in vitro and in vivo (Thanou et al., 2002). Various chitosans and its derivatives have been reported for gene delivery. However, the toxicity of those chitosans was different depending on the type of cells and derivatives studied. Therefore, the cytotoxicity study of the chitosan derivatives/DNA complex was performed in Huh7 cells. Fig. 6 shows the effect of TM_{57}-Bz_{18}-CS /DNA (Fig. 6a), TM_{47}-Bz_{42}-CS/DNA (Fig. 6b) and TM_{57}-Bz_{42}-CS/DNA complexes (Fig. 6c) on cell viability. When Huh7 cells were incubated with 1 μg of naked DNA, cell viability remained almost the same as that seen in control non-transfected cells (data not shown). There was significant

decrease in cell viability when Huh7 cells were incubated with various weight ratios of TM-Bz-CS/DNA complexes. The average cell viability decreased when the weight ratios increased. However, the viability was over 80% at the weight ratio from which the highest transfection efficiency was obtained. It was clear that TM-Bz-CS/DNA complexes were safe at the concentration used.

4. Conclusions

In this study, the novel water soluble and high transfection efficiency chitosan derivatives (TM-Bz-CS) were successfully synthesized by chemically modified with hydrophobic moiety of dimethylaminobenzyl and by *N*-quaternization. This study indicated that the degree of dimethylaminobenzyl substitution (DS) was crucial for high transfection efficiency.

Acknowledgments

The authors acknowledge the financial support from the Research, Development and Engineering (RD&E) Fund through National Nanotechnology Center (NANOTEC), Thailand (Project No. NN-B-22-b3-17-49-07), Thailand Graduate Institute of Science and Technology Scholarship (TGIST) and the Thailand Research Funds (Project No. DBG5180005).

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International Journal of Pharmaceutics 382 (2009) 291–295

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Nuclear localization signal peptides enhance transfection efficiency of chitosan/DNA complexes

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ARTICLE INFO

Article history:
Received 29 June 2009
Received in revised form 4 August 2009
Accepted 23 August 2009
Available online 28 August 2009

Keywords:
Nuclear localization signal
Chitosan
Gene delivery
Transfection efficiency

ABSTRACT

The purpose of this study was to investigate the potential of nuclear localization signal (NLS) "KPKKKRKV" to mediate the in vitro transfection efficiency of chitosan (CS)/DNA complexes, aiming at its use in gene therapy applications. In the preparation of CS/DNA complexes containing NLS, peptide with NLS was directly incorporated without covalent conjugation to pDNA or chitosan. The gene transfer efficiency of CS/DNA complexes with and without NLS was evaluated in the human cervical carcinoma cell line (Hela cells). The CS/DNA complex containing NLS increased transfection efficiencies in a NLS-dose dependent manner on the Hela cells, compared to the control (CS/DNA complex or NLS). The highest transfection efficiency was significantly observed in CS/DNA complex at the weight ratio of 8 with 120 μg NLS and was 74-fold higher than that in the cells transfected with CS/DNA complex. Cytotoxicity of the NLS/CS/DNA complexes increased as the amount of the peptide increased, however, over 80% average cell viability was observed for complexes at the effective concentration of the peptide for transfections. Therefore, the NLS is expected to be a potent transfection enhancing agent without a covalent conjugation to pDNA or chitosan. Our findings suggest that the high gene expression with the negligible cytotoxicity can be achieved by adding the NLS peptide to chitosan/DNA complexes at an optimal ratio.

1. Introduction

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. The main objective in gene therapy is successful in vivo transfer of the genetic materials to the targeted tissues. However, naked therapeutic genes are rapidly degraded by nucleases and show poor cellular uptake, so the development of safe and efficient gene carriers is one of the prerequisites for the success of gene therapy (Rolland, 2005). One approach is a non-viral delivery system based on supramolecular assembly. Cationic lipids and cationic polymers have been employed as non-viral gene transfer agents. These cationic substances form complexes with anionic DNA by electrostatic interaction. The resultant cationic DNA complexes were taken up by cells through electrostatic interaction because the cell surface is negatively charged. Among those, cationic liposomes are widely used for almost all animal cells because they have nonspecific ionic interaction and low toxicity (Ruozi et al., 2003). However, there are some limitations since when they are used for in vivo transfection, they are unstable. Therefore, many polymeric cationic systems such as polyethyleneimine (PEI), cationic peptides (poly L-lysines; PLL), cationic dendrimers and chitosan have been studied for in vitro as well as in vivo application (Tang et al., 2006; Tiera et al., 2006).

Chitosan (CS) is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) produced by alkaline deacetylation of chitin. CS is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values, but soluble in acidic medium. CS is biocompatible, biodegradable and non-toxic; therefore, it has been proposed as a safer alternative to other non-viral vectors (Ishii et al., 2001; Weecharangsan et al., 2006). Formulation parameters such as molecular weight (MW), degree of deacetylation (DD), N/P ratio (ratio of positively charged chitosan to negatively charged DNA), and pH of transfection medium were found to affect the transfection efficiency of CS/DNA complexes (Huang et al., 2005; Lavertu et al., 2006; Weecharangsan et al., 2008). The main drawback of CS is the poor water solubility at physiological pH and low transfection efficiency. Several CS derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics. Modified CS such as glycol CS or PEGylated CS and quaternized CS, low MW soluble CS, and salt forms could be

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doi:10.1016/j.ijpharm.2009.08.029

possible ways to circumvent the solubility issues. To improve gene transfection, chemically modified CS was reported (Kim et al., 2003; Chae et al., 2005; Lee et al., 2007; Opanasopit et al., 2008, 2009).

Recent studies indicated that the nuclear membrane is a serious barrier to the delivery and expression of exogenous gene and thus many attempts have been made to overcome this barrier. Among various approaches, the use of nuclear localization signal (NLS) peptides for non-viral gene transfer has been widely investigated (Dean et al., 1999; Escriou et al., 2003; Prasad and Rao, 2005). Nuclear pore complexes (NPCs) control the passage of molecules in and out the nucleus. The inner pore of a NPC allows free, passive diffusion of molecules of up to 9 nm in diameter. Additionally an active transport of small molecules up to 25 nm is possible (Ludtke et al., 1999). Larger molecules require so-called NLS that are recognised by cytoplasmatic transport receptors and mediate the nuclear uptake. NLS sequences are typically less than 12 residues in length and rich in basic amino acids resulting in a net positive charge. The most studied and therefore best known NLS sequences are peptides derived from viruses like Tat (transactivating) protein or *Antennapedia homeodomain* protein derived ones, but arginine/lysine-rich NLS such as for the simian virus 40 (SV40) large T antigen (PKKKRKV) seem to be far more efficient than those peptides. The various approaches explored differ mainly in the method used for the covalent (Ludtke et al., 1999; Prasad and Rao, 2005; Bremner et al., 2004) or non-covalent (Chan and Jans, 1999; Cartier and Reszka, 2002) attachment of the NLS peptide(s) to DNA and in the use of linear DNA or circular (plasmidic) DNA. In the case of NLS covalently attached to the DNA, care has to be taken to avoid binding of the NLS within the expression cassette, hence blocking subsequent transcription of the transgene. While many of these approaches have met with limited success, a significant (10–1000-fold) enhanced gene expression was obtained following ligation of a NLS-oligonucleotide conjugate to one or both ends of a linear DNA (Zanta et al., 1999). Other approach of NLS covalent attachment is to attach with gene carriers. Jeon et al. showed that the covalent conjugation of a NLS (SV40 peptide) on poly(L-lactide-co-glycolide) nanospheres enhanced the gene transfection efficiency (Jeon et al., 2007). Recently, NLS was chemically conjugated to a DNA intercalating reagent. Yoo et al. revealed that the DNA/PEI complex consisting NLS attached to psoralen, a nucleic acid-intercalating agent increased transfection efficiencies on COS-1 cells (Yoo and Jeong, 2007). On the other hand, Boulanger et al. reported that the expression of the transgene in most cases did not improve upon complexation of plasmid DNA with NLS-acridine conjugates prior to its formulation as lipoplexes or polyplexes (Boulanger et al., 2005).

The ionic residues of NLS-containing peptide can also interact with the polycations typically used for DNA condensation or the hydrophobic residues with lipid components. The NLS can therefore influence the physical properties of the heteroplex such as particle size, DNA condensation rate, surface charge distribution and intracellular stability, which eventually influence the transfection efficiency. In this study, we determined physicochemical properties of CS/DNA complexes such as particle size and charge with and without NLS. A number of variables that influenced transfection efficiency such as CS/DNA N/P ratio, amount of NLS, pH of culture medium and serum were investigated. Based on these data, we elucidated the main factors influencing the enhanced transfection efficiency in vitro.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Seafresh Chitosan Lab., Thailand with MW of 45 kDa and 87% degree of deacetylation. Chitosan salt (CS; chitosan HCl) was prepared as previously described (Weecharangsan et al., 2008). Briefly, chitosan was dissolved in distilled water containing hydrochloric acid at 1:1 molar ratio. The solution was stirred for 12 h and spray-dried. Polyethylenimine (PEI), MW 25 kDa, was purchased from Aldrich, Germany. The NLS peptide (KPKKKRKV) was synthesized by Peptide Synthesis Department, Bio Basic Inc. Ontario, Canada. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM), trypsin–EDTA, penicillin–streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen, USA. The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, USA. The λHindIII were obtained from Promega, USA. Huh7 (Human hepatocellular carcinoma) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

3. Methods

3.1. Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA). DNA concentration was quantified by the measurement of UV absorbance at 260 nm using a GeneRay UV Photometer (Biometra®, Göttingen, Germany). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate-EDTA (TAE) buffer, pH 8.0 using λDNA/HindIII as a DNA marker.

3.2. Preparation and characterization of CS/DNA complexes with and without NLS

The CS/DNA complexes were prepared at various N/P ratios by adding the DNA solution to the CS solution. The mixture was gently mixed using pipette for 3–5 s to initiate complex formation and then NLS solution was added and left for 15 min at room temperature. The complex formation was confirmed by electrophoresis. Agarose gels were prepared with 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/ml). The electrophoresis was carried out for 60 min at 100 V. The volume of the sample loaded in the well was 15 μl of CS derivatives/DNA complex containing 1 μg of DNA.

3.3. Size and zeta potential measurements

The particle size and surface charge of CS/DNA complexes with and without NLS were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior used. All samples were measured in triplicate.

3.4. Morphology

The morphology of the CS/DNA complexes was determined by Atomic Force Microscope (AFM, SPI4000-SPA400, Chiba, Japan) using tapping-mode AFM in air. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior used. These samples were dropped immediately onto freshly cleaved mica and dry in air.

3.5. In vitro transfection

Hela cells were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 ml of growth medium (DMEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air, 37 °C) for 24 h. Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). The cells were incubated with 0.5 ml of the CS/DNA complexes with and without NLS at various N/P ratios containing 1 μg of pDNA for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes were used as controls. After transfection, the cells were washed twice with PBS and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

3.6. Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. Hela 7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μl of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to transfection, the medium was removed and the cells were rinsed with PBS, and then supplied with the CS/DNA complexes with and without NLS in the same concentrations as in vitro transfection experiment. After treatment, CS/DNA complexes solutions were removed. Finally, the cells were incubated with 100 μl MTT containing medium (1 mg/ml) for 4 h. Then the medium was removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μl DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

3.7. Statistical analysis

Statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < 0.05$.

4. Results and discussion

4.1. Characterization of CS/DNA complexes with and without NLS

In order to determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between CS and DNA at different CS concentrations with and without NLS. The formation of complexes between CS and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. By varying the concentration of CS and fixing the DNA concentration, ratios of the weight of positively charged CS (due to amine groups) to negatively charged DNA (due to the phosphate groups), which were referred as weight ratio of the particle formulations were varied. Fig. 1 shows DNA marker (lane 1), the naked DNA (lane 2) and CS/DNA complexes at weight ratios of 0.5, 1, 2, 3, 4, 8 and 16 (lanes 3–9). The naked DNA showed the DNA bands, whereas complexed DNA was retained within the gel loading well for CS at weight ratio above 2, illustrating that complete CS/DNA complexes were formed. In contrast to the preparation of CS/DNA complexes containing 120 μg NLS peptides, it was found that complexes could be completely formed at all ratios. These indicated that the binding between CS and DNA was dependent on NLS. NLS sequences are rich in basic amino acids resulting in a net positive charge that facilitated the complexation between CS and DNA (Bremner et al., 2004).

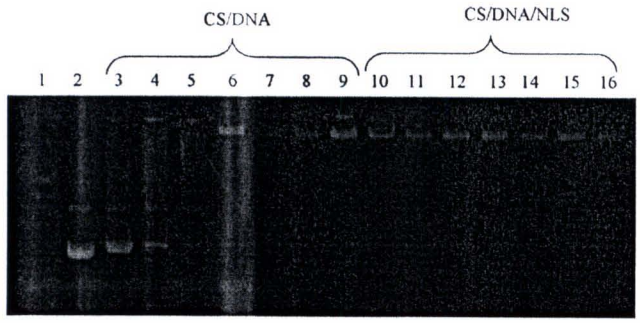

Fig. 1. Gel retarding analysis of CS/DNA complexes formulated with and without NLS. Lane 1, DNA marker; lane 2, pEGFP-C2 plasmid, lanes 3–9 (CS/DNA complexes) and lanes 10–16 (CS/DNA complexes with 120 μg of NLS) at weight ratios of 0.5, 1, 2, 3, 4, 8 and 16, respectively.

Particle size and the zeta potential of various weight ratios of CS/DNA complexes with and without NLS were shown in Table 1. The particle size of CS/DNA complexes slightly increased with the increasing weight ratio from 2 to 24 in the range of 300–500 nm. The zeta potential of the complexes was found to increase with the increase in weight ratios of CS/DNA complexes due to their higher density of protonated amines in the CS backbone. A similar result was observed in CS/DNA with NLS. At low amount of CS (2–4 μg), an addition of NLS resulted in increase in the particle size. On the other hand, at the higher amount of CS (8–24 μg), an addition of NLS resulted in decrease in the particles size. Moreover, the addition of NLS yielded the increase in the zeta potential. These results indicated that an addition of NLS affected both particle size and surface charge of CS/DNA complexes. The morphological examination of the CS/DNA complexes at a weight ratio of 4 with 120 μg NLS was performed by AFM. The AFM images (Fig. 2) revealed that the complexes were spherical with nanosize.

4.2. In vitro transfection

The achievement of high gene transfection efficiency is a final goal for the development of novel gene carriers. To investigate the NLS mediated gene transfection efficiencies, in vitro gene transfection assay was performed with Hela cells using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). In order to investigate the optimal conditions for gene transfection, CS/DNA complexes

Table 1
Particle size and zeta potential of CS/DNA complexes with and without NLS.

CS (μg)	NLS (μg)	DNA (μg)	Particle size (nm)	Zeta potential (mV)
0	120	1	ND	ND
2	0	1	332 ± 27	3.7 ± 1.5
	120	1	603 ± 25	10.4 ± 1.5
4	0	1	410 ± 12	6.2 ± 2.5
	60	1	542 ± 7	12.1 ± 5.5
	120	1	495 ± 41	11.9 ± 5.1
	160	1	639 ± 27	11.2 ± 3.4
	200	1	580 ± 50	13.4 ± 1.9
	240	1	676 ± 26	15.6 ± 0.7
8	0	1	423 ± 18	8.1 ± 1.6
	120	1	408 ± 26	13.6 ± 3.1
16	0	1	507 ± 17	8.4 ± 0.8
	120	1	255 ± 14	14.3 ± 3
24	0	1	397 ± 40	9.3 ± 1.7
	120	1	185 ± 6	13.6 ± 3.2

ND = not determined.

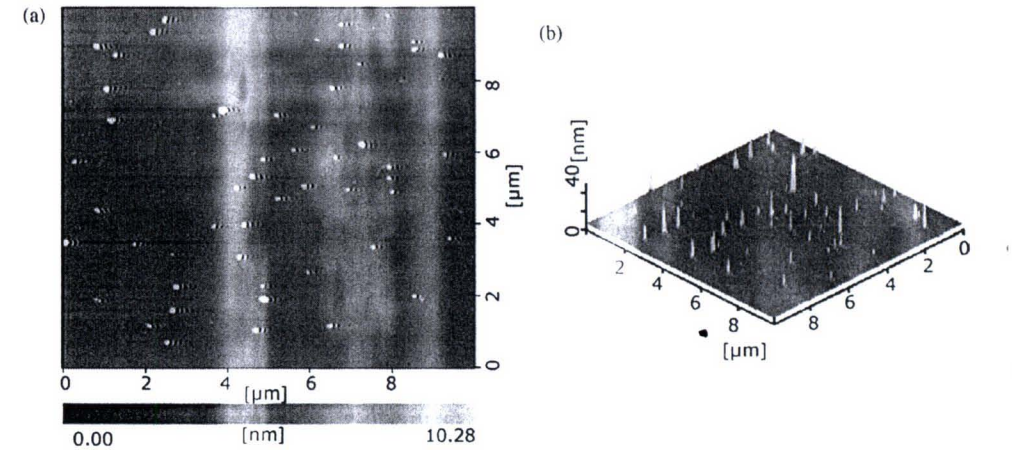

Fig. 2. The atomic force microscope (AFM) images of CS/DNA complexes at weight ratio of 4 mixed with 120 μg of NLS: (a) planar image and (b) three-dimensional image.

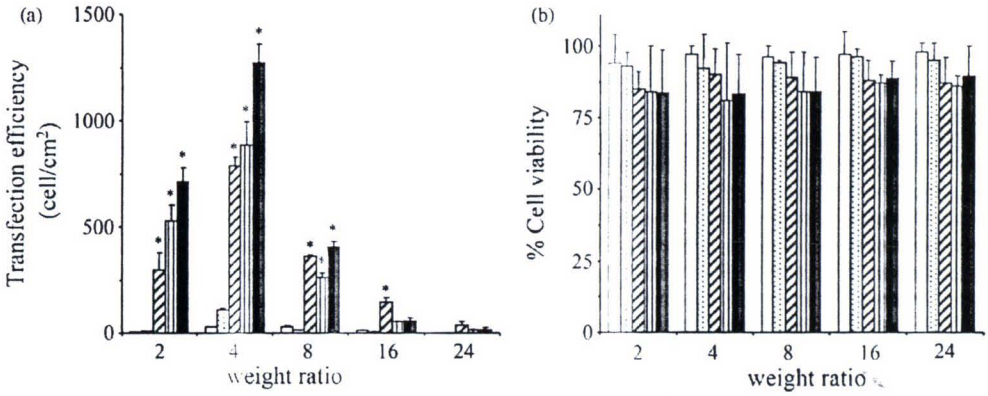

Fig. 3. (a) Transfection efficiencies and (b) cell viability of CS/DNA complexes at varying N/P ratios formulated with NLS: 0 μg (□), 30 μg (▨), 60 μg (▨), 90 μg (▨) and 120 μg (■) in Hela cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

were formulated with various weight ratios (2, 4, 8, 16 and 24) with and without NLS. Polyethylenimine (PEI, 25 kDa) complexed with DNA at the weight ratio of 1 was used as a positive control and had the gene transfection of 600 cell/cm^2. In all studies, there were no transfection in control (cells without complexes), naked DNA and NLS/DNA complexes with various weight ratios (30, 60, 90, 120 and 240). As shown in Fig. 3 (at pH 6.2), the gene transfection efficiencies were significantly influenced by the CS/DNA ratios and amount of NLS. By increasing the ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. The highest transfection efficiency was observed at the weight ratios of CS/DNA complex of 4 with the addition of 120 μg NLS (Fig. 3a). Fig. 4a represents the influence of NLS amount (0–240 μg) on transfection efficiency of CS/DNA complex at the weight ratio of 4. The transfection efficiencies increased when the amount of NLS increased and reached the maximum at 120 μg. Then, the transfection efficiencies decreased by further increment of the NLS. These results revealed that not only the CS/DNA complex ratio but also the amount of NLS affected the gene transfection efficiency. Our results clearly demonstrate that ternary complexes,

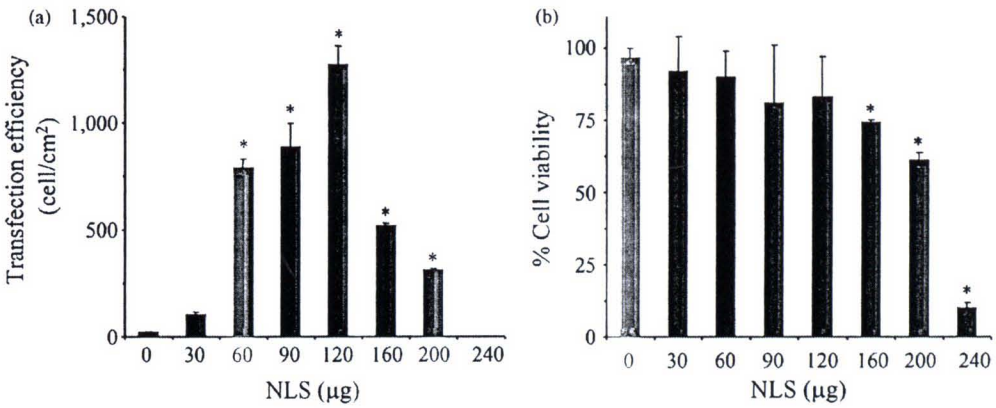

Fig. 4. (a) Transfection efficiencies and (b) cell viability of CS/DNA complexes at weight ratio of 4 formulated with various amount of NLS in Hela cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

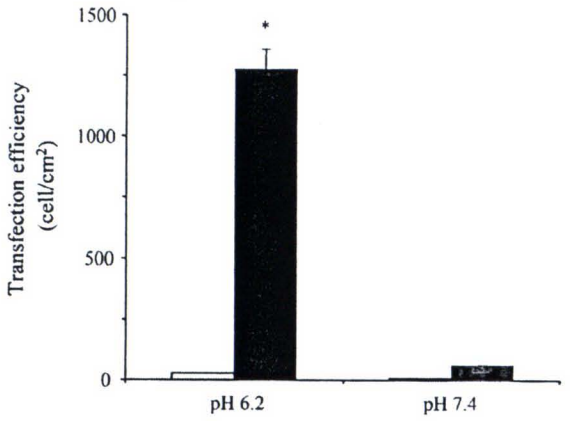

Fig. 5. Effect of pH medium at pH 7.4 and pH 6.2 with NLS 120 μg (■) and without NLS (□), on transfection efficiencies of CS/DNA complexes at the weight ratio of 4 in Hela cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < 0.05$).

resulting from the association of NLS to CS/DNA complexes, are significantly more efficient in mediating transfection than the corresponding NLS/DNA or CS/DNA complexes. Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 (Weecharangsan et al., 2008). As shown in Fig. 5, pH dramatically affected both CS and CS with NLS with decreasing the transfection efficiency by increasing pH values from 6.2 to 7.4. Although the exact mechanism of NLS mediated efficient gene delivery remains to be further studied, our results clearly demonstrate that the addition of NLS could thereby influence the physical properties of the heteroplex such as particle size, surface charge distribution, DNA condensation, and intracellular stability. These effects could influence the transfection efficiency of the complex and could be potential candidate for non-viral gene carriers.

4.3. Effect of NLS/CS/DNA complexes on cell viability

One of the major requirements for cationic polymer vectors for gene delivery is low cytotoxicity. It has been reported that CS were less toxic than other cationic polymers such as poly-lysine and PEI in vitro and in vivo (Lavertu et al., 2006). However, the toxicity of association of NLS to CS/DNA complexes was not studied. Therefore, the cytotoxicity study of NLS/CS/DNA complex was conducted in Hela cells. Fig. 3b shows the effect of NLS/CS/DNA complex on cell viability. There was significant decrease in cell viability when Hela cells were incubated with increasing amount of NLS in all CS/DNA ratios. However, the viability was over 80% at the addition of 120 μg NLS where the highest transfection efficiency was obtained. By further increment of the NLS more than 120 μg, % cell viability was dramatically decreased (Fig. 4b) resulting in decrease the transfection efficiency. A similar result was observed in previous studies (Trabulo et al., 2008).

5. Conclusion

Our results clearly demonstrate that ternary complexes, resulting from association of NLS to CS/DNA complexes, are significantly more efficient in mediating transfection than the corresponding NLS/DNA or CS/DNA complexes. Cytotoxicity of the NLS/CS/DNA complexes increased as the amount of the peptide increased, however, over 80% average cell viability was observed for complexes at the effective concentration of the peptide for transfections. Our findings suggest that the high gene expression can be achieved by adding NLS peptide to CS/DNA complexes at optimal ratio.

Acknowledgments

The authors would like to acknowledge the financial support from Silpakorn University Research and Development Institute (SURDI 52/01/30), Commission of Higher Education (Thailand) and the Thailand Research Fund (Project No. DBG5180005).

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OLIGONUCLEOTIDES
Volume 20, Number 3, 2010

DOI: 10.1089/oli.2009.0227

Nucleic Acid Delivery with Chitosan Hydroxybenzotriazole

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The objective of this study was to investigate the transfection efficiency of chitosan hydroxybenzotriazole (CS-HOBT) for *in vitro* nucleic acid delivery. The results revealed that CS-HOBT was able to condense with DNA/small interfering double-stranded RNA molecules (siRNA). Illustrated by agarose gel electrophoresis, complete complexes of CS-HOBT/DNA were formed at a weight ratio of above 3, whereas those of CS-HOBT/siRNA were formed at a weight ratio of above 4 (CS molecular weights [MWs] 20 and 45 kDa) and above 2 (CS MWs 200 and 460 kDa). Gel electrophoresis results indicated that binding of CS-HOBT and DNA or siRNA depended on the MW and weight ratio. The particle sizes of CS-HOBT/nucleic acid complexes were in nanosize range. The highest transfection efficiency of CS-HOBT/DNA complex was found at a weight ratio of 2, with the lowest CS MW of 20 kDa. The CS-HOBT-mediated siRNA silencing of the enhanced green fluorescent protein gene occurred maximally with 60% efficiency. The CS-HOBT/siRNA complex with the lowest CS MW of 20 kDa at a weight ratio of 80 showed the strongest inhibition of gene expression. For cytotoxicity studies, over 80% the average cell viabilities of the complexes were observed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This study suggests that CS-HOBT is straightforward to prepare, is safe, and exhibits significantly improved nucleic acid delivery potential *in vitro*.

Introduction

GENE DELIVERY is one of the critical steps for gene therapy. Viral vectors have been proven to have high gene transfection efficiency. However, they have some disadvantages such as immunogenicity and potential of insertional mutagenesis in the host genome (Lundstrom, 2003). In light of safety concerns, nonviral delivery systems have been developed. Among those, polymers are the major type of nonviral vectors investigated in the past decade (Midoux et al., 2008, 2009; Jere et al., 2009). Many polymeric cationic systems such as gelatin, polyethyleneimine (PEI), poly(L-lysines), tetraminofullerene, poly(L-histidine)-*graft*-poly(L-lysines), Diethylaminoethyl–Dextrans, cationic dendrimers, cationic polyrotaxanes, and chitosan (CS) and its derivatives have been studied for *in vitro* as well as *in vivo* application (Dang and Leong, 2006; Tang et al., 2009; Yang et al., 2009).

RNA interference represents a powerful tool for the silencing of specific genes. This process is mediated via small interfering double-stranded RNA molecules (siRNAs) that sequence-specifically trigger the cleavage and subsequent degradation of target mRNA. It is a posttranscriptional gene silencing process mediated by a 21–23-mer duplex siRNA that has a sequence homologous to the silenced gene (Sharp, 2001). For therapeutic applications, siRNA technology promises greater advantages over drugs that are currently on the market by offering new types of drugs that are easy to design and have very high target selectivity (Dorsett and Tuschl, 2004; Ryther et al., 2005). Therapeutics using siRNA, however, are hindered by poor intracellular uptake and limited blood stability of siRNA. Many delivery systems of siRNA based on physical and pharmaceutical approaches have been proposed. The two main types of vectors used in gene therapy (viral and nonviral vectors) are also reported for siRNA delivery (Landen et al., 2005; Urban-Klein et al., 2005).

CS is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine produced by alkaline deacetylation of chitin. CS is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values, but soluble in acidic medium. CS has been used as a vector for gene and siRNA delivery. In addition, CS is biocompatible, biodegradable, and nontoxic; therefore, it

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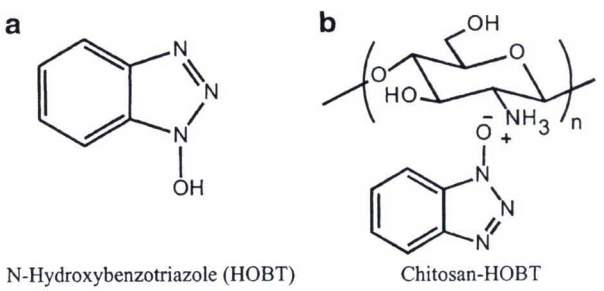
has been proposed as a safe alternative to other nonviral vectors such as cationic lipids and cationic polymers (Ishii et al., 2001; Lee et al., 2001; Weecharangsan et al., 2006, 2008). At acidic pH, below pK_a, the primary amines in the CS backbone become positively charged. These protonated amines enable CS to bind to negatively charged DNA or siRNA and condense DNA or siRNA into particles. Moreover, CS has shown capability to protect DNA from DNase I and II degradation (Zhao et al., 2006). Formulation parameters such as molecular weight (MW), degree of deacetylation, ratio of positively charged CS to negatively charged DNA, and pH of transfection medium were found to affect the transfection efficiency of CS/DNA (Kiang et al., 2004; Huang et al., 2005; Lavertu et al., 2006) or CS/siRNA complexes (Howard et al., 2006; Katas and Alpar, 2006; Pille et al., 2006; Liu et al., 2007). Recently, our research group found that CS can easily dissolve in thiamine pyrophosphate solution and this CS–thiamine pyrophosphate-mediated siRNA silencing of the endogenous enhanced green fluorescent protein (EGFP) gene occurred maximally with 70%–73% efficiency, which was higher than Lipofectamine 2000™ (Rojanarata et al., 2008).

Hydroxybenzotriazole (HOBT) is an organic compound used as a racemization suppressor and to improve yield in peptide synthesis. CS-HOBT aqueous solution prepared by simply mixing CS and HOBT in water provides an effective system to functionalize CS in an aqueous environment (Fangkangwanwong et al., 2006). The chemical structure of HOBT is shown in Figure 1a. Because of the OH groups of HOBT, the molecule can form salt with amine groups of CS and improve CS water solubility (Fig. 1b). In this study, the CS salts formed between CS with different MWs (20, 45, 200, and 460 kDa) and HOBT were evaluated for their ability to form complexes with plasmid DNA encoding EGFP (pEGFP-C2) and siRNA. The transfection efficiency of CS-HOBT/DNA was examined in human hepatoma cell lines (Huh7 cells). The transfection efficiency of CS-HOBT/siRNA complexes was examined in human hepatocarcinoma cell lines (HepG2 cells), stably expressing EGFP. Particle size and zeta potential of CS-HOBT/DNA and CS-HOBT/siRNA complexes were evaluated. In addition, the cytotoxicity of CS-HOBT/DNA and CS-HOBT/siRNA complexes was investigated.

Materials and Methods

Materials

CS was purchased from Seafresh Chitosan Lab, with MWs 20, 45, 200, and 460 kDa and 85% degree of deacetylation.



N-Hydroxybenzotriazole (HOBT) Chitosan-HOBT

FIG. 1. Chemical structure of (**a**) *N*-hydroxybenzotriazole (HOBT) and (**b**) chitosan-HOBT (CS-HOBT).

HOBT and PEI (branched; MW 25 kDa) were purchased from Aldrich. Agarose, diethylpyrocarbonate (DEPC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma. Modified Eagle's medium (MEM), fetal bovine serum (FBS), trypsin–ethylenediaminetetraacetic acid (EDTA), and penicillin–streptomycin were purchased from Gibco BRL. siRNA-EGFP(+) and siRNA-EGFP(−) were synthesized by using Ambion's Silencer™ siRNA Construction Kit (Ambion). Human hepatocarcinoma cell lines (HepG2 cells) and human hepatocellular carcinoma (Huh7 cells) were obtained from American Type Culture Collection. The pEGFP-C2 plasmid DNA, encoding GFP, was obtained from Clontech. All other chemicals were of cell culture and molecular biology quality.

Preparation of CS-HOBT

An aqueous CS-HOBT solution was prepared at a CS: HOBT molar ratio of 1:1, as reported previously (Fangkangwanwong et al., 2006). Briefly, HOBT H_2O (9.4 mg, 0.06 mmol) was dissolved in 10 ml distilled water, and CS (10 mg, 0.06 mmol) was added to the HOBT solution and vigorously stirred with a magnetic stirrer at ambient temperature until the solution became clear.

Plasmid DNA preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen). DNA concentration was quantified by the measurement of UV absorbance at 260 nm using a GeneRay UV Photometer (Biometra®). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate-EDTA buffer, pH 8.0, using λDNA/*Hin*dIII as a DNA marker.

siRNA preparation

The EGFP-targeted siRNA, siRNA-EGFP(+), and the mismatch siRNA, siRNA-EGFP(−), were synthesized using Ambion's Silencer siRNA Construction Kit (Ambion). The duplex siRNA-EGFP(+) contains sense 5′-gcu gac ccu gaa guu cau cuu-3′ and antisense 5′-gau gaa cuu cag ggu cag cuu-3′. The siRNA-EGFP(−) contains sense 5′-gca ccg cuu acg uga uac uuu-3′ and antisense 5′-agu auc acg uaa gcg gug cuu-3′. The siRNA-EGFP(+) targets to position 124–144 of EGFP open-reading frame and siRNA design suggested by the company. The mismatch siRNA-EGFP(−) was designed by scrambling the nucleotide sequence of siRNA-EGFP(+) and performing blast analysis to identify the sequences that lack significant homology to human genes.

Complex formation between CS-HOBT and pDNA or siRNA

Complexes of CS-HOBT/pDNA and CS-HOBT/siRNA were prepared at various CS-HOBT/DNA or siRNA weight ratios by adding the pDNA or siRNA solution to the CS-HOBT solution and diluting with water and 0.1% DEPC water, respectively. The mixture was gently pipetted and vortexed for 3–5 seconds to initiate complex formation and left at room temperature for 30 minutes to complete the process. Complex formation was confirmed by electrophoresis. Agarose gels were prepared with 0.8% agarose solution for CS-HOBT/

pDNA complexes and 2% agarose solution for CS-HOBT/siRNA complexes in Tris-borate-EDTA buffer with ethidium bromide (0.2 μg/ml). The electrophoresis was carried out for 20 minutes at 100 V in Tris-borate-EDTA running buffer. The volume of the sample loaded in the well was 10 μl of complex containing 1 μg of DNA or 140 ng of siRNA and 2 μl of 50% glycerol in water. The DNA or siRNA in the complexes were visualized under a UV transilluminator.

Size and zeta potential measurement

The particle sizes and surface charge of the CS-HOBT/pDNA and CS-HOBT/siRNA complexes were measured by using the Zetasizer Nano ZS (Malvern Instruments Ltd.) at room temperature. The complexes were diluted with water for pDNA or 0.1% DEPC water for siRNA, which were passed through a 0.22-μm membrane filter prior to use. All samples were measured in triplicate.

In vitro *transfection of Huh7 cells with CS-HOBT/DNA complexes*

Huh7 cells were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 ml of growth medium (Dulbecco's MEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% nonessential amino acid solution, 100 U/ml penicillin, and 100 μg/ml streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air, 37°C) for 24 hours. Before transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS; pH 7.4). The cells were incubated with 0.5 ml of the CS-HOBT/DNA complex at various weight ratios containing 1 μg of pDNA for 24 hours at 37°C under 5% CO_2 atmosphere. Nontreated cells and cells transfected with naked plasmid and PEI/DNA complexes were used as controls. After transfection, the cells were washed twice with PBS and grown in culture medium for 48 hours to allow for GFP expression. All transfection experiments were performed in triplicate.

HepG2 cells stably express EGFP

HepG2 cells with stable constitutive expression of EGFP were generated as described previously (Rojanarata et al., 2008). Briefly, HepG2 cells were transfected with a pEGFP-C2 plasmid (Clontech) using Lipofectamine 2000 (Invitrogen). The cells were trypsinized and diluted to get single cells. The cells were treated with 0.5 mg/ml G418 to generate clones of stable cell lines. Individual clones were isolated and expanded in MEM, supplemented with 10% FBS, and treated with 0.1 mg/ml G418 every 3–4 weeks to maintain the EGFP gene expression.

In vitro *transfection of HepG2 stable cells with CS-HOBT/siRNA complexes*

The HepG2 cells stably expressing EGFP were cultivated in MEM, supplemented with 10% FBS, in a humidified atmosphere (5% CO_2, 95% air, 37°C). On the day of transfection, the trypsinized cells were suspended in MEM at a concentration of 2.5×10^5 cells/ml. The CS-HOBT/siRNA (EGFP-specific or EGFP-mismatch) complexes at various weight ratios were formed for 30 minutes by mixing equal volumes of siRNA and CS-HOBT diluted in 0.1% DEPC water. A volume of 100 μl of cell suspension was mixed with 20 μl of CS-HOBT/siRNA complexes (350 ng/well siRNA) in a sterile Eppendorf® tube. A volume of 120 μl of cell suspension was then transferred into black clear-bottomed 96-well plates (Corning, USA) at the density of 25,000 cells/well. After two 2 hours of transfection, the cells were adhered to the well. For the initial fluorescent intensity used to calculate % seeding variation, measurement of EGFP was performed using a fluorescence microplate reader (Universal Microplate Analyzer, models AOPUS01 and AI53601,; Packard BioScience, CT, USA) with excitation/emission at 485/530 nm. To ensure the same number of cells in each well, we first calculated % seeding variation (Eq. 1) and then used the values to adjust the fluorescent intensity obtained from each well by Eq. 2. The variation of the fluorescence intensity of each well was less than 10%. After 24 hours of transfection, the medium was removed and the cells were washed with PBS. Following this, the media were replaced with fresh medium. The plates were incubated for five 5 days at 37°C and 5% CO_2, measuring the fluorescence intensity daily. Percentage of inhibition of green fluorescence was calculated using Eq. 3.

$$\%\ \text{Seeding variation} = \frac{(I_{\text{average,day0}} - I_{n,\text{day0}})\times 100}{I_{\text{average,day0}}} \quad (1)$$

$$\text{Adjusted fluorescent intensity } (I_{\text{ad}}) = I_{n,\text{d1-4}} + \frac{[(I_{n,\text{d1-4}})\times(\%\ \text{seeding variation})]}{100} \quad (2)$$

$$\%\ \text{Inhibition of gene expression} = \frac{(I_{\text{ad,siRNA(-)}} - I_{\text{ad,siRNA(+)}})\times 100}{I_{\text{ad,siRNA(-)}}} \quad (3)$$

where $I_{\text{average,day0}}$ is the average fluorescent intensity of all wells at 2 hours after transfection (day 0), $I_{n,\text{day0}}$ is the fluorescent intensity of individual well at day 0, $I_{n,\text{d1-4}}$ is the fluorescent intensity at days 1 through 5 of each well, I_{ad} is the adjusted fluorescent intensity, $I_{\text{ad,siRNA(+)}}$ is the adjusted fluorescent intensity of the cell with CS/siRNA(+) complexes, and $I_{\text{ad,siRNA(-)}}$ is the adjusted fluorescent intensity of the cell with CS/siRNA(−) complexes.

Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. In CS-HOBT/DNA complexes, Huh7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μl of growth medium and incubated for 24 hours at 37°C under 5% CO_2 atmosphere. In CS-HOBT/siRNA complexes, HepG2 cells stably expressing EGFP were seeded and transfected using the same method as the *in vitro* transfection. After 24 hours of transfection, the medium was removed and the cells were washed with PBS. Following this, 20 μl of MTT (5 mg/mL) was added to each well and the incubation was continued for 4 hours. The medium was then removed, the cells were rinsed with PBS (pH 7.4), and formazan crystals that had formed in the living cells were dissolved in 100 μl of dimethyl sulfoxide per well. Relative viability (%) was calculated based on absorbance at 550 nm, using a microplate reader (Universal Microplate Analyzer, models AOPUS01

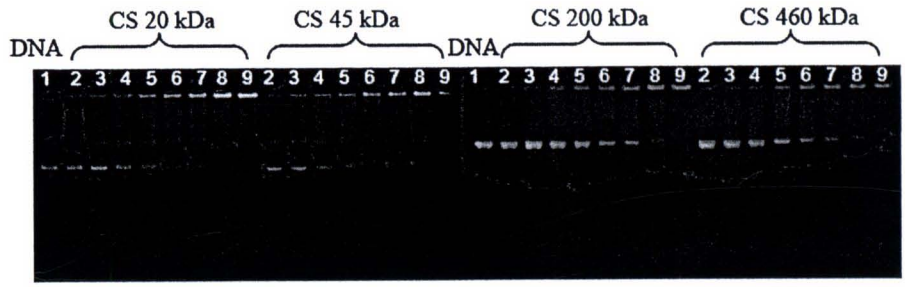


FIG. 2. Gel retarding analysis of CS-HOBT/DNA complexes formulated with CS MWs 20, 45, 200, and 460 kDa on 0.8% agarose gel. Lane 1, plasmid DNA encoding enhanced green fluorescent protein (pEGFP-C2); lanes 2–9, CS-HOBT/DNA complexes at weight ratios of 0.01, 0.1, 0.5, 1, 1.5, 2, 3, and 4, respectively.

and AI53601; Packard BioScience). Viability of nontreated control cells was arbitrarily defined as 100%.

Statistical analysis

Statistical significance of differences in transfection efficiency and cell viability was examined using one-way analysis of variance, followed by an LSD *post hoc* test. The significance level was set at $p < 0.05$.

Results

Characterization of CS-HOBT/DNA complexes

To determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between CS-HOBT and DNA at different CS concentrations. The formation of complexes between CS-HOBT and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. Figure 2 shows the naked DNA (lane 1) and CS-HOBT/DNA complexes at weight ratios 0.01, 0.1, 0.5, 1, 1.5, 2, 3 and 4 (lanes 2–9). To investigate the effect of MW on complex formation, CS-HOBT/DNA complexes with different MWs of CS (20, 45, 200, and 460 kDa) were formulated. The naked DNA lane showed the DNA band, whereas complexed DNA was completely retained within the gel-loading well for all MWs of CS at weight ratios above 3, illustrating that complete CS-HOBT/DNA complexes were formed.

Particle size and zeta potential of CS-HOBT/DNA complexes at various weight ratios formulated with CS MWs 20, 45, 200, and 460 kDa are shown in Table 1. The particle size of the CS-HOBT/DNA complexes decreased to constant value in the range of 200–300 nm at a weight ratio of 2 for CS MW 20 kDa. Higher CS MWs (45, 200, and 460 kDa) had higher particle size and zeta potential than lower CS MW of 20 kDa. An initial negative value of zeta potential was observed at a low weight ratio of 0.5. At the weight ratios greater than 0.5, the zeta potential reached a plateau of about +20 to +51 mV depending on the MW of CS.

Characterization of CS-HOBT/siRNA complexes

To investigate the optimal conditions in forming CS-HOBT/siRNA complexes, it is necessary to evaluate the degree of binding between CS-HOBT and siRNA at different MWs of CS and weight ratios. The complexes were formed at an acidic pH of 4.6 in DEPC-treated water. The formation of complexes between CS-HOBT and siRNA was observed by using the modified method of Grayson et al. (2006) based on gel retardation on 2% agarose gel. Figure 3 shows the naked siRNA and CS-HOBT/siRNA complexes at weight ratios 0.25, 0.5, 1, 2, 4, 8, 16, and 32 when different MWs of CS were used. The results showed that siRNA binding to CS-HOBT was dependent on both the MW of CS and the weight ratio. At a weight ratio of less than 8 (Fig. 3, lanes 2–6), the migration behavior was almost the same as the siRNA control (Fig. 3, lane 1). The gradual retardation effect on the migration of siRNA was observed when the weight ratio increased. When the MW of CS increased, a greater retardation effect on the migration of siRNA was observed. The complexes were completely retained within the well at a weight ratio above 2 (lane 5) for high MWs of 200 and 460 kDa and above 4 (lane 6) for low CS MWs of 20 and 45 kDa.

The physical properties including size and surface charge, which influence cellular interactions and nanoparticle distribution, were measured. Particle size and zeta potential of CS-HOBT/siRNA complexes at various weight ratios formulated

TABLE 1. PARTICLE SIZE AND ZETA POTENTIAL OF CHITOSAN/DNA COMPLEXES

	CS MW 20 kDa		*CS MW 45 kDa*		*CS MW 200 kDa*		*CS MW 460 kDa*	
CS/DNA weight ratio	*Particle size (nm)*	*Zeta potential (mV)*	*Particle size (nm)*	*Zeta potential (mV)*	*Particle size (nm)*	*Zeta potential (mV)*	*Particle size (nm)*	*Zeta potential (mV)*
0.5	479 ± 20	−15.7 ± 1.1	749 ± 93	−13.8 ± 0.5	586 ± 36	−13.2 ± 1.1	794 ± 88	−9.2 ± 0.5
1	335 ± 30	20.2 ± 1.5	324 ± 23	24.5 ± 0.6	758 ± 60	23.1 ± 2.7	452 ± 16	30.9 ± 1.5
2	246 ± 24	25.2 ± 1.3	325 ± 63	29.4 ± 0.8	488 ± 19	50.7 ± 4.7	430 ± 42	51.5 ± 2.4
3	279 ± 15	29.6 ± 2.3	452 ± 70	33.7 ± 3.1	579 ± 16	51.7 ± 1.1	722 ± 79	49.1 ± 2.8
4	285 ± 23	32.5 ± 1.1	300 ± 51	36.3 ± 2.7	581 ± 19	53.9 ± 1.8	733 ± 20	51.2 ± 2.7

CS, chitosan; MW, molecular weight.

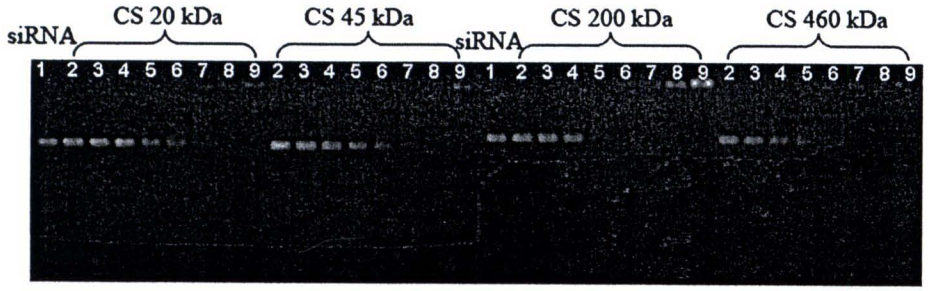


FIG. 3. Gel retarding analysis of CS-HOBT/small interfering double-stranded RNA molecule (siRNA) complexes formulated with CS MWs 20, 45, 200, and 460 kDa on 2% agarose gel. Lane 1, siRNA-EGFP; lanes 2–9, CS-HOBT/DNA complexes at weight ratios of 0.25, 0.05, 1, 2, 4, 8, 16, and 32, respectively.

with CS MWs 20, 45, 200, and 460 kDa are shown in Table 2. Zeta potential value increased with increasing weight ratio. MW of CS showed no effect on surface charge and size of complexes. The range of zeta potential was -6.2 ± 0.2 to 49.1 ± 2.1 mV. The increment was due to the increase in the number of positive charges of the CS. The particle sizes of CS-HOBT/siRNA complexes at a weight ratio of 20–80 were in nanosize range (157 ± 45 to 719 ± 72 nm).

In vitro *transfection of Huh7 cells with CS-HOBT/DNA complexes*

The achievement of high gene transfection efficiency is the final goal for the development of novel gene carriers. To investigate the CS-HOBT-mediated gene transfection efficiencies, *in vitro* gene transfection assay was performed with human hepatoma cell lines (Huh7 cells) using pEGFP-C2 plasmid encoding GFP. PEI (25 kDa) was used as a positive control. The transfection efficiency of PEI/DNA complexes at a weight ratio of 1 was 900 cells/cm^2. CS-HOBT/DNA complexes were formulated with various MWs of CS (20, 45, 200, and 460 kDa) to investigate the effect of MW on transfection efficiency. The transfection efficiencies of CS-HOBT/DNA complexes are shown in Figure 4a. In all studies, there were no transfection in control (cells without complexes) and naked DNA. As shown in Figure 4a, the gene transfection efficiencies at pH 6.4 were significantly influenced by the CS-HOBT/DNA ratios and MW of CS. By increasing the ratios, the transfection efficiencies reached the highest values, with a decrease by further increment of the ratios. The highest transfection efficiencies of all MWs of CS-HOBT/DNA complexes were at a weight ratio of 2. Among the CS MWs, 20 kDa showed the highest transfection efficiency, followed by 45, 200, and 460 kDa, respectively.

Previous studies reported that the transfection efficiency of CS was dependent on pH and serum. CS-mediated high gene transfection was observed at the medium pH values below 6.5 (Weecharangsan et al., 2008). Cationic liposome-associated gene expression was inhibited by serum, whereas CS showed resistance to serum (Sato et al., 2001). As shown in Figure 4b, CS-HOBT-associated gene expression was inhibited at physiological pH 7.4 and in the presence of 10% serum. Increasing pH from 6.4 to 7.4 dramatically decreased the transfection efficiency. The presence of 10% serum in the transfection medium with pH 7.4 also decreased the transfection efficiency.

In vitro *transfection of HepG2 stable cells with CS-HOBT/siRNA complexes*

The HepG2 cells that stably express EGFP were used as a model system. Lipofectamine 2000 was used as a positive control. Figure 5 shows the reduction in EGFP expression in stable constitutive EGFP-HepG2 cells treated with CS-HOBT/siRNA complexes at 1–4 days posttransfection. The concentration of siRNA was kept constant at 350 ng/well and the weight ratio was varied from 0.4 to 80. A negligible gene silencing effect was observed for naked siRNA. Complexes of CS-HOBT/siRNA containing EGFP-mismatch siRNA [siRNA-EGFP(−)] showed no EGFP inhibition, confirming knockdown specificity. The effect of posttransfection time on the gene silencing efficiency is shown in Figure 5. In CS-HOBT/siRNA complexes, the silencing efficiency was increased with the increase of posttransfection time. The posttransfection time of 4 days was therefore chosen for comparison of the gene silencing efficiency of Lipofectamine 2000/siRNA (positive control) and CS-HOBT/siRNA at different weight ratios (Fig. 6). The silencing efficiencies were

TABLE 2. PARTICLE SIZE AND ZETA POTENTIAL OF CHITOSAN/SMALL INTERFERING DOUBLE-STRANDED RNA MOLECULE COMPLEXES

	CS MW 20 kDa		*CS MW 45 kDa*		*CS MW 200 kDa*		*CS MW 460 kDa*	
CS/siRNA weight ratio	*Particle size (nm)*	*Zeta potential (mV)*	*Particle size (nm)*	*Zeta potential (mV)*	*Particle size (nm)*	*Zeta potential (mV)*	*Particle size (nm)*	*Zeta potential (mV)*
0.4	452 ± 60	-2.03 ± 0.1	1171 ± 84	-3.8 ± 0.1	2282 ± 171	-0.3 ± 1.1	1494 ± 87	-6.2 ± 0.2
20	342 ± 30	43.4 ± 1.8	324 ± 56	36.9 ± 1.7	471 ± 70	45.0 ± 1.5	370 ± 15	41.9 ± 1.1
40	165 ± 24	25.5 ± 2.1	320 ± 83	35.6 ± 1.5	417 ± 46	46.1 ± 4.2	428 ± 45	41.5 ± 2.4
80	157 ± 45	39.9 ± 4.1	459 ± 76	45.3 ± 3.1	651 ± 76	42.9 ± 1.5	719 ± 72	49.1 ± 2.1

siRNA, small interfering double-stranded RNA molecules.

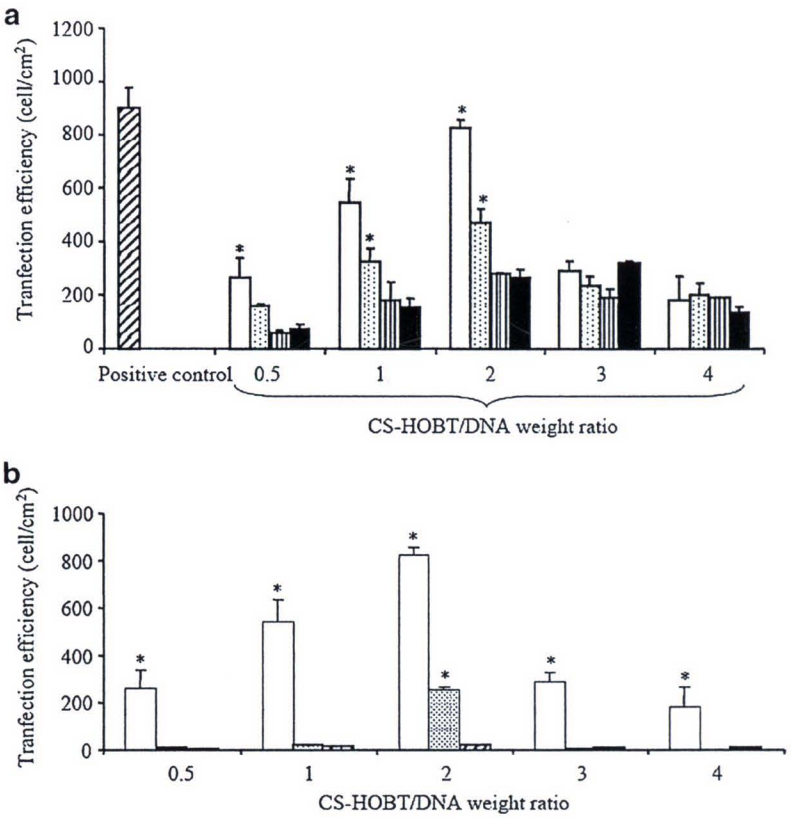


FIG. 4. Comparison of the transfection efficiencies of (**a**) polyethyleneimine/DNA (positive control; bar with crossed lines) and CS-HOBT/DNA complexes formulated with CS MWs 20 kDa (empty bar), 45 kDa (dotted bar), 200 kDa (bar with vertical lines), and 460 kDa (black bar) at various weight ratios in Huh7 cells. (**b**) Effect of pH medium and serum on transfection efficiencies of CS-HOBT 20 kDa/DNA complexes at pH 6.4 without serum (empty bar), pH 7.4 without serum (dotted bar), and pH 7.4 with 10% serum (bar with crossed lines) in Huh7 cells. Each value represents the mean ± standard deviation (SD) of three wells. *Difference values were statistically significant ($p < 0.05$).

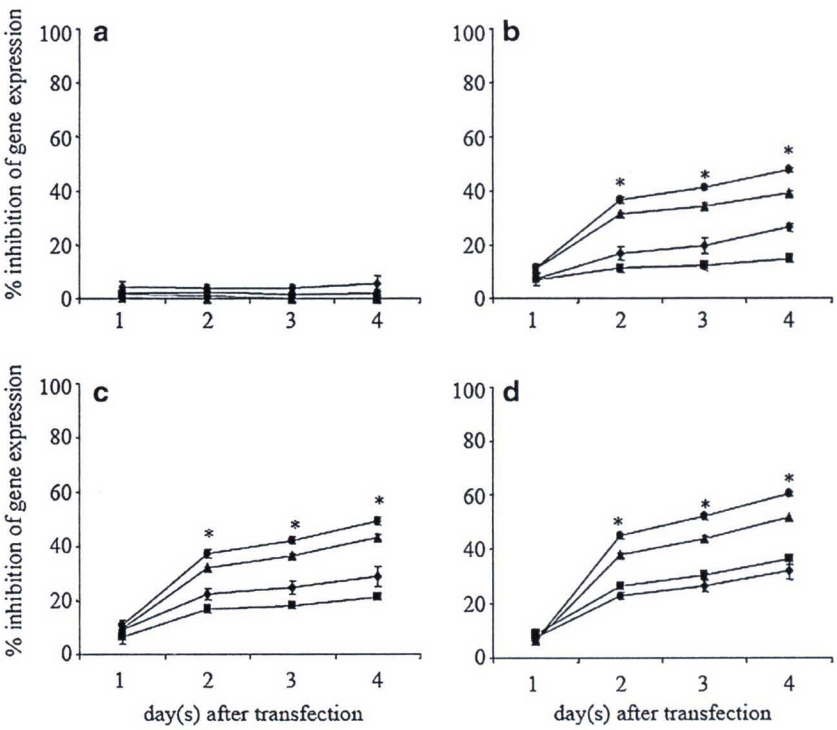


FIG. 5. Effect of weight ratio and molecular weight of CS on gene silencing. Percentage of EGFP gene knockdown by CS-HOBT/siRNA complexes at various weight ratios (**a**) 0.4, (**b**) 20, (**c**) 40 and, (**d**) 80 in HepG2 cells stably expressing EGFP at 1–4 days posttransfection. CS MWs were 20 kDa (●), 45 kDa (▲), 200 kDa (◆), and 460 kDa (■). Data are plotted in mean ± SD of eight wells. *Difference values were statistically significant ($p < 0.05$).

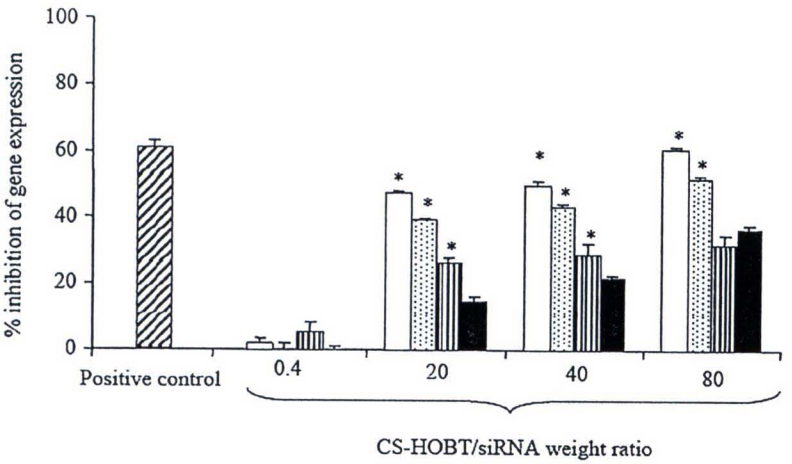


FIG. 6. Comparison of the percentage of EGFP gene knockdown by Lipofectamine 2000™/siRNA (positive control; bar with crossed lines) and CS-HOBT/siRNA at various weight ratios in HepG2 cells stably expressing EGFP at 4 days posttransfection at pH 7.2. CS MWs were 20 kDa (empty bar), 45 kDa (dotted bar), 200 kDa (bar with vertical lines), and 460 kDa (black bar). Data are plotted as mean ± SD of eight wells. *Difference values were statistically significant ($p < 0.05$).

significantly influenced by weight ratios, which gradually increased as the weight ratio increased. The highest gene silencing level (60% inhibition) was reached at a weight ratio of 80 and a CS MW of 20 kDa. At a weight ratio of 20, 40, and 80, the gene silencing efficiency of CS-HOBT had a tendency to decrease as the MW increased. At different MWs, the maximum gene silencing efficiency was found at a CS MW of 20 kDa. These results indicated that both the MW of CS and the weight ratio of CS/siRNA complexes are the important formulation parameters that affect siRNA delivery.

Cytotoxicity

The cytotoxicity of CS has been reported (Carreño-Gómez and Duncan, 1997). However, there was no report of CS-HOBT. Therefore, the CS-HOBT/DNA complexes and CS-HOBT/siRNA complexes were investigated for a possible cytotoxic effect. The cytotoxicity of CS/DNA complexes and CS/siRNA complexes was found to be low compared with other cationic complexes (Lavertu et al., 2006; Weecharangsan et al., 2006; Rojanarata et al., 2008). To investigate the potential cytotoxicity of CS-HOBT/DNA complexes and CS-HOBT/siRNA complexes, cell viability was determined by MTT assay after 24 hours incubation with complexes. The observed relative cytotoxicities of CS are shown in Figure 7. There was significant decrease in cell viability when Huh7 cells were incubated with various weight ratios of CS-HOBT/DNA complexes (Fig. 7a). The average cell viability decreased when the CS-HOBT/DNA weight ratios increased. However, the viability was over 80% at a weight ratio of 2, at which the highest transfection efficiency was obtained. On the other hand, the cytotoxicity of CS-HOBT/siRNA complexes showed that CS-HOBT/siRNA complexes formulated with various MWs of CS (20, 45, 200, and 460 kDa) at various weight ratios of 0.4, 20, 40, and 80 were not significantly different from the untreated cells. Average cell viability was over 80% (Fig. 7b). Therefore, from this study, the CS-HOBT has been clearly proved to be safe.

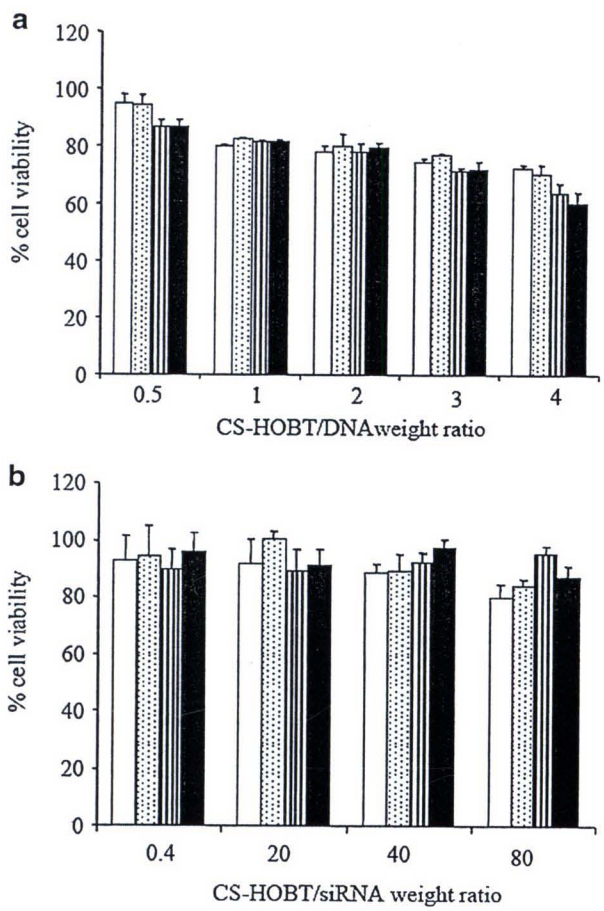


FIG. 7. Cell viability of (a) CS-HOBT/DNA complexes at various weight ratios in Huh7 cells and (b) CS-HOBT/siRNA complexes at various weight ratios in HepG2 cells stably expressing EGFP. CS MWs were 20 kDa (empty bar), 45 kDa (dotted bar), 200 kDa (bar with vertical lines), and 460 kDa (black bar). Each value represents mean ± SD of six wells.

Discussion

CS has been used to formulate for gene delivery (Kiang et al., 2004; Huang et al., 2005; Lavertu et al., 2006) and siRNA delivery (Katas and Alpar, 2006; Howard et al., 2006; Pille

et al., 2006; Liu et al., 2007; Rojanarata et al., 2008; Mao et al., 2009). However, the effect of HOBT salt forms of CS on transfection efficiency has not been elucidated. In this study, an aqueous CS-HOBT solution was easily prepared at a CS:HOBT molar ratio of 1:1 by dissolving CS with HOBT solution. CS-HOBT/DNA complexes formulated with different MWs of CS (20, 45, 200, and 460 kDa) were investigated for their transfection abilities. The pEGFP-C2 plasmid was used as the reporter gene to monitor the transfection efficiency in Huh7 cells. Moreover, the transfection efficiency of CS-HOBT/siRNA complexes was examined in HepG2 cells, which stably express EGFP. It was found that CS can easily dissolve in HOBT solution and CS-HOBT could sufficiently transfect Huh7 cells and this CS-HOBT-mediated siRNA silencing of the EGFP gene occurred maximally with 60% efficiency with low cytotoxicity.

Analysis by gel electrophoresis indicated that CS-HOBT formulated with CS-HOBT has abilities to form complexes with DNA and siRNA. As our previous result, it was demonstrated that the degree of binding between CS and DNA was largely dependent on the pH of the complex solution, which possibly affected the degree of protonation of amine groups in CS and the weight ratio (Weecharangsan et al., 2008). The MW of CS also affected complex formation (Huang et al., 2005; Lavertu et al., 2006). In this study, the CS-HOBT/DNA complexes were preformed in water (pH 6.4). The result revealed that complexed DNA was completely retained within the gel-loading well for all MWs of CS at weight ratios above 3. The MW of CS slightly affected the binding, whereas the weight ratio of CS/DNA largely affected the binding. The particle size of all CS-HOBT/DNA complexes increased with increasing weight ratio. This was due to intermolecular crosslinking between DNA strands by self-aggregates with an excess amount of CS. The increase in particle size of CS/DNA complexes with increasing CS was also observed in a previous report (MacLaughlin et al., 1998). At an acidic pH (pH 6.4), the transfection efficiency was affected by CS MW and weight ratio. The maximum transfection efficiency of all MWs of CS-HOBT/DNA complexes was at a weight ratio of 2. Cell viability at ratios of 3 and 4 was a little lower than that at 2 and the particle sizes were almost comparable for the complexes prepared at ratios from 2 to 4. This could be due to an optimal association and dissociation between CS and DNA in the CS/DNA complexes with an optimal MW of CS and weight ratio of CS/DNA complexes, which resulted in high gene expression efficiency (Lavertu et al., 2006). Among the MWs of CS, 20 kDa showed the highest transfection efficiency, followed by 45, 200, and 460 kDa, respectively. This could be due to the high association between CS and DNA, which prevented dissociation once inside the cells. This observation is in agreement with previous studies (MacLaughlin et al., 1998; Sato et al., 2001; Mao et al., 2009). On the other hand, high transfection efficiency of CS/DNA complexes formulated with high-MW CS was observed by Zhao et al. (2006). In comparison, transfection efficiency of CS-HOBT/DNA at a weight ratio of 2 was 2.5 times higher in gene transfection than that of CS lactate at a MW of 20 kDa (Weecharangsan et al., 2006). These results revealed that the counterion HOBT salt enhanced the gene transfection efficiency. Previous studies reported that the transfection efficiency of CS was dependent on pH and serum (Weecharangsan et al., 2008). Our results also showed that CS-HOBT-associated gene expression was inhibited at physiological pH 7.4 and in the presence of 10% serum. CS at an acidic pH has shown promise in protecting DNA from DNase I and II degradation (Zhao et al., 2006) and in transfecting into different cell types. At an acidic pH below pK_a, the primary amines in the CS backbone become positively charged. These protonated amines enable CS to bind negatively charged DNA or siRNA and condense it into particles. With a pK_a value of the D-glucosamine residue of approximately 6.2–7.0, CS is a weak base and is insoluble under neutral and alkaline conditions.

In siRNA, the formation of complexes between CS-HOBT and the siRNA in DEPC-treated water (pH 4.6) was dependent on both the MW of CS and the weight ratio. The gradual retardation effect on the migration of siRNA was observed when the weight ratio increased. This result indicated that the high-MW CS bound siRNA slightly better than the low-MW CS. The influence of MW on complex formation, as explained previously, could be attributed to a chain entanglement effect because longer CS chains in high-MW CS more easily entangle and trap free siRNA than shorter chain abundantly found in low-MW CS (Liu et al., 2007; Rojanarata et al., 2008). Liu et al. (2007), using a polyanion displacement method, revealed that siRNA was rapidly displaced from the CS/siRNA complexes upon addition of a polyanion (poly-L-aspartic acid). In comparison, the gel analysis of CS-HOBT/siRNA and CS-HOBT/DNA complexes indicated that DNA and siRNA bind similarly to CS. DNA and siRNA completely condensed to the CS at a weight ratio above 3 and above 2–4, respectively. *In vitro* transfection of CS-HOBT/siRNA complexes in HepG2 stable cells showed that the silencing efficiency was increased with increase in posttransfection time (Fig. 5). The silencing efficiencies were significantly influenced by weight ratios, which gradually increased as the weight ratio increased. The highest gene silencing level (60% inhibition) was reached at a weight ratio of 80 and a CS MW of 20 kDa. These results indicated that both the MW of CS and the weight ratio of CS-HOBT/siRNA complexes are the important formulation parameters that affect siRNA delivery. The CS-HOBT/siRNA complexes achieved sufficient transfection efficiencies at higher weight ratios. This might be due to the reason that an increase in the concentration of CS at higher weight ratios could yield a higher amount of complete complexes and had positively charged complexes to successfully transfect cells. This event was inconsistent with results found with DNA. In DNA, high transfection efficiency required a lower weight ratio of 2. This might be due to different interactions between CS and siRNA, which were apparently weak, as CS and siRNA are easily dissociated from each other compared with CS associated with plasmid DNA at the same weight ratio. High transfection occurred in low-MW CS (20 kDa). These might be due to the reasons that complexes formed with large number of short-chain CS, were abundantly found in low-MW CS, and released siRNA better than the complexes formed with long chain of high-MW CS.

Conclusion

Successful nucleic acid delivery was observed in cell culture by using a water-soluble CS salt (CS-HOBT). The ability of this CS salt to transfer functionally active nucleic acid into cell culture is dependent upon the weight ratio and MW of CS.

This study suggests that CS-HOBT is simple to prepare, is safe, and exhibits significantly improved nucleic acid delivery potential *in vitro*.

Acknowledgments

The authors are grateful for financial support by the National Nanotechnology Center (NANOTEC), Thailand (grant number NN-B-22-EN3-17-52-10), the Commission of Higher Education (Thailand), and the Thailand Research Funds (grant number: DBG5180005).

Author Disclosure Statement

No competing financial interests exist.

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Received for publication December 3, 2009; accepted after revision February 7, 2010.

International Journal of Pharmaceutics xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Chitosan enhances transfection efficiency of cationic polypeptides/DNA complexes

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ARTICLE INFO

Article history:
Received 27 January 2011
Received in revised form 1 March 2011
Accepted 6 March 2011
Available online xxx

Key words:
Cationic polypeptides
Poly-L-arginine
Chitosan
Gene delivery

ABSTRACT

The aim of this research was to investigate the effect of cationic polypeptides mixed with chitosan (CS) on in vitro transfection efficiency and cytotoxicity in human cervical carcinoma cells (HeLa cells). The polypeptides/DNA complexes and ternary complexes (CS, polypeptides and DNA) at varying weight ratios were formulated and characterized by using gel electrophoresis. Their particle sizes and charge were evaluated. The effect of the type and molecular weight (MW) of polypeptides, the weight ratio, order of mixing, the pH and serum on transfection efficiency and cytotoxicity were evaluated in HeLa cells. Three types of polypeptides (poly-L-lysine; PLL, poly-L-arginine; PLA and poly-L-ornithine; PLO) were able to form complete complex with DNA at weight ratio above 0.1. The PLA MW >70 kDa showed the highest transfection efficiency. The order of mixing between CS, PLA and DNA affected the transfection efficiency. The highest transfection efficiency was observed in ternary complexes of PLA/DNA/CS (2:1:4) equal to PEI/DNA complex. For cytotoxicity studies, over 80% the average cell viabilities of the complexes were observed by MTT assay. This study suggests that the addition of CS to PLA/DNA is easy to prepare, safe and exhibits significantly improved DNA delivery potential in vitro.

1. Introduction

Gene therapy requires safe and efficient vehicles to transfer and deliver expressible genetic material to target tissues. The most extensively used delivery tools are viral-based vectors, which are highly effective. In this type of approach, the viruses can act so as to sneak foreign genes into cells. Unfortunately, viruses, even disabled ones, can cause serious side effects (Lundstrom, 2003). The second approach involves using a nonviral vector such as cationic lipids and cationic polymers (Wong et al., 2007; Midoux et al., 2008, 2009; Ruozi et al., 2003). Many polymeric cationic systems such as gelatin, polyethyleneimine (PEI), poly(L-lysines), tetraminofullerene, poly(L-histidine)-graft-poly(L-lysines), diethylamino ethyl-dextrans, cationic dendrimers, cationic polyrotaxanes and chitosan and its derivatives have been studied for in vitro as well as in vivo application (Dang and Leong, 2006; Weecharangsan et al., 2006; Opanasopit et al., 2008, 2009; Kim et al., 2003a; Chae et al., 2005). Chitosan [(1 → 4) 2-amino-2-deoxy-β-D-glucan] is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) produced by alkaline deacetylation of chitin. Chitosan is a weak base with a pKa value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values, but soluble in acidic medium such as acetic acid, citric acid, glutamic acid, aspartic acid, hydrochloric acid and lactic acid.

Cationic polypeptides have been used as tools for gene carriers. The main reason for the using cationic polypeptides is that cationic polymers vectors provide the flexibility of DNA carrying capacity and simplicity of use. The nature of the basic peptide in efficient protein transduction and in nucleic acid binding capability made them possible to be the carrier for DNA transfection (Saccardo et al., 2009; Ferrer-Miralles et al., 2008). The most used DNA-condensing cationic peptides in gene delivery systems are poly-L-lysines (PLL) and related peptides. The peptides can bind the negatively charged backbone of DNA chain, not only promoting its condensation but also favoring the interaction of the nanoparticle with the cell membrane and the consequent internalizations. However, the degree of polymerization has shown to be directly related to toxic effects, the longer the lysine chain, the more the cytotoxic PLL (Martin and Rice, 2007; Plank et al., 1999). The cationic poly-L-arginine (PLA) and poly-L-ornithine (PLO) has been used in the recent years as an alternative to lysine in non-viral gene delivery systems (Tecle et al., 2003). From the study of polypeptide/DNA complexes, the physicochemical properties and the transfection efficiency of the complexes could be resulted from the effect of type and MW of polypeptide. In addition, it has been reported that the biological activities of the transfection reagents are highly associated with their physicochemical properties (Thomas and Klibanov, 2003). The variable type and MW of polypeptide, weight ratio and pH

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doi:10.1016/j.ijpharm.2011.03.008

of transfection medium influenced the physicochemical properties and transfection efficiency of polypeptide/DNA complexes (Pouton and Seymour, 1998; Ramsay et al., 2000; Choi et al., 2006; Goparaju et al., 2009).

Recently, cationic polypeptide was reported to be able to translocate through cell membrane very easily, and these cell penetrating peptides (CPPs) containing arginines and lysines, have been used as DNA and oligo DNA carriers (Takeuchi et al., 2006). Arginine residues were finally applied to polymeric gene delivery carriers. These arginine residues have been conjugated to poly (amido amine) (PAMAM) dendrimer (Nam et al., 2008), poly (N-isopropylacrylamide) (Cheng et al., 2006) and chitosan (Gao et al., 2008), and they showed significantly higher transfection efficiency than unmodified polymers. Therefore, combining the unique properties of biodegradable polymers such as chitosan with the advantages of cationic polypeptides (PLL, PLO and PLA), we expect that this self-assembly vector was easy to prepare without any chemical synthesis and would show high transfection efficiency with its low cytotoxicity. In this study, cationic polypeptides/DNA complexes and ternary complexes (CS, PLA and DNA) were prepared and their physicochemical properties intended for gene delivery such as the complex formation, particle size and zeta potential of complexes were investigated with the complex solution of different type and MW of polypeptides, weight ratio and pH of transfection medium. A number of variables influencing transfection efficiency and cell cytotoxicity such as carrier/DNA weight ratio, type and molecular weight of polypeptides, order of mixing, pH of culture medium and serum were also investigated.

2. Materials and methods

2.1. Materials

Poly-L-arginine hydrochloride (PLA; MW 5–15, 15–70 and >70 kDa), poly-L-ornithine hydrobromide (PLO; MW 30–70 kDa), poly-L-lysine hydrobromide (PLL; MW 30–70 kDa) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Polyethylenimine (PEI; MW 25 kDa) was purchased from Aldrich (Milwaukee, USA). Chitosan with MW of 45 kDa and 87% degree of deacetylation was purchased from Seafresh Chitosan Lab., Thailand. Chitosan salt (CS; chitosan HCl) was prepared as previously described (Weecharangsan et al., 2008). Briefly, chitosan was dissolved in distilled water containing hydrochloric acid at 1:1 molar ratio. The solution was stirred for 12 h and spray-dried. MEM medium, Trypsin-EDTA, penicillin-streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen (Grand Island, NY, USA). The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, USA. The λ*Hin*dIII were obtained from Promega (Madison, WI, USA). HeLa (human cervical carcinoma) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

2.2. Methods

2.2.1. Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA). DNA concentration was quantified by the measurement of UV absorbance at 260 nm using a GeneRay UV Photometer (Biometra®, Göttingen, Germany). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate-EDTA (TAE) buffer, pH 8.0 using λDNA/*Hin*dIII as a DNA marker.

2.2.2. Complex formation of polypeptides/DNA

The polypeptides/DNA complexes were prepared at various weight ratios by adding the DNA solution to the cationic polypeptide solution, then mixed by pipetting up and down and tapping the tubes gently. They were incubated for 30 min at room temperature to ensure complex formation.

2.2.3. Ternary complexes formation of CS and PLA/DNA

In this study, CS and DNA were fixed at 4 μg and 1 μg, respectively, with various weight ratios of PLA MW >70 kDa (0–50) and the order of mixing. Three type of order of mixing use in this study were: (1) adding the DNA solution to PLA solution and then added CS solution (PLA/DNA/CS); (2) adding the DNA solution to CS solution and then added PLA solution (CS/DNA/PLA); and (3) adding the PLA solution to CS solution and then added DNA solution (PLA/CS/DNA). These mixtures were gently mixed using pipette for 3–5 s to initiate complex formation and then left for 30 min at room temperature.

2.2.4. Characterization of complexes

The complex formation was confirmed by electrophoresis (MyRun intelligent, Cosmo Bio, Japan). Agarose gels were prepared with 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/ml). The electrophoresis was carried out for 60 min at 100 V. The volume of the sample loaded in the well was 15 μl of complex containing 1 μg of DNA.

2.2.5. Size and zeta potential measurements

The particle size and surface charge of CS/DNA complexes with and without polypeptide were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior to use. All samples were measured in triplicate.

2.2.6. Binding affinity

Ethidium bromide (EtBr) displacement assay was performed in order to study the ability of CS and polypeptide to bind with DNA. EtBr (0.1 mg/ml) was dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.4). 20 μl of EtBr solution was added to a 20 μl of 500 μg/ml DNA solution. The steady state fluorescent measurement was performed on a spectrofluorometer (RF-1501, Shimadzu, Tokyo, Japan) at an excitation wavelength of 560 and an emission wavelength of 605 nm. An aliquot CS solution (1 mg/ml) or PLA (1 mg/ml) was then titrated into the DNA/EtBr solution to the varied weight ratios of DNA/CS/PLA complexes (1:4:0 to 50) with varying order of mixing. The fluorescent intensity calculated based on the fluorescent intensity of the DNA/EtBr solution is shown in Eq. (1). The recorded fluorescent intensity (FI) was expressed relative to the fluorescent intensity of the DNA/EtBr solution in the absence of CS or polypeptide (FI_0), after subtracting the fluorescence of EtBr in the absence of DNA under the same buffer conditions (FI_{buff}). Data are presented as mean ± SD. The assay was performed in triplicate.

$$FI\ (\%) = \left[\frac{FI - FI_{buff}}{FI_0 - FI_{buff}}\right] \times 100 \quad (1)$$

2.2.7. In vitro transfection

HeLa cells were seeded into 24-well plates at a density of 4×10^4 cells/well in 1 ml of growth medium (MEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air, 37 °C) for 24 h. Prior to transfection, the medium was removed and the cells were rinsed with PBS, pH 7.4. The cells were incubated with

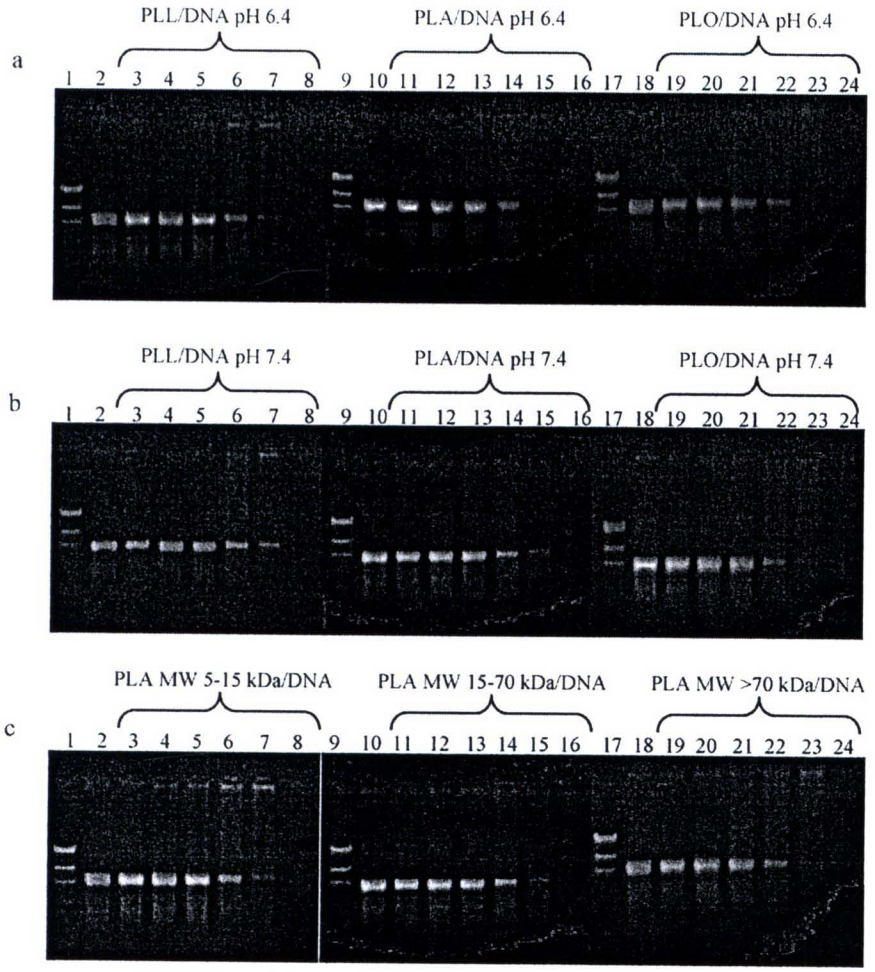

Fig. 1. Gel retarding analysis of polypeptides/DNA complexes (a) at pH 6.4, (b) at pH 7.4 and (c) PLA/DNA complexes with different molecular weight at pH 7.4. Lanes 1, 9, 17 DNA marker; lanes 2, 10, 18 pEGFP-C2 plasmid; lanes 3–8, 11–16, 19–24 of polypeptides/DNA complexes at weight ratios of 0.001, 0.005, 0.01, 0.05, 0.1 and 0.5, respectively.

0.5 ml of the polypeptides/DNA complexes or ternary complexes of CS and PLA/DNA at various weight ratios and order of mixing containing 1 μg of pDNA for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes at the weight ratio of 1 (yielding the maximum transfection efficiency) were used as controls. After transfection, the cells were washed twice with PBS and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

2.2.8. Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. HeLa cells were seeded in a 96-well plate at a density of 8×10^3 cells/well in 100 μl of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to evaluation, the medium was removed and the cells were rinsed with PBS, and then supplied with the polypeptides/DNA complexes or ternary complexes of CS and PLA/DNA at various weight ratios and order of mixing in the same concentrations as in vitro transfection experiment. After treatment, the complexes solutions were removed. Finally, the cells were incubated with 100 μl MTT containing medium (1 mg/ml) for 4 h. Then the medium was removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μl DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

2.2.9. Statistical analysis

Statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Characterization of polypeptide/DNA complexes

In order to determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between polypeptide and DNA at different polypeptide type and molecular weight. The formation of complexes between polypeptide and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. The results showed that three types of cationic polypeptides (PLL, PLA and PLO), MW of PLA (5–15, 15–70 and >70 kDa), and pH (6.4 and 7.4) did not affect the complex formation (Fig. 1). The complete complexes were formed at weight ratio above 0.1. These results indicated that DNA binding to polypeptides was independent from the type, molecular weight of polypeptides and pH of complexes solution.

Accordingly, the complete complexes were able to form at the weight ratio above 0.1, regardless to the type of polypeptide. To deliver DNA into cells, the particle size and zeta potential of a complex are the important factors that influence the access and passage of the complex through the targeting site. The effect of pH on the particle size and zeta potential of polypeptide/DNA complexes were investigated in the complex solution at pH 6.4 and 7.4, and the results are shown in Table 1. It was found that their particle size and zeta potential were dependent on the weight ratio. The particle size of three cationic polypeptides (PLL, PLA and PLO)/DNA complexes at pH 7.4 were in nanometer (130–560 nm) and slightly decreased with an increasing weight ratio from 1 to 4. Whereas the zeta-potential of these complexes slightly increased with an increasing weight ratio from 1 to 4 and had the positive charge in the range of +15 to +30 mV. In addition, as the MW of PLA increased, the particle size of the complexes had a trend showing a slightly increase (data not shown). The particle size of PLL/DNA and PLA/DNA complexes at pH 6.4 was a slightly larger than those at pH 7.4. However at pH 6.4, the all complexes were nanosize (200–800 nm). The particle sizes of PLO/DNA complexes were slightly larger than PLL/DNA and PLA/DNA complexes. The charge densities of polypeptides increase when the pH of the polypeptide solution decreases from high to low pH. Due to the increase charge density of polypeptide at low pH, the slight increase of particle size could be resulted from the exclusion of redundant positive charge provided by polypeptide inside nanoparticles.

3.2. Characterization of ternary complexes of CS and PLA/DNA

Physicochemical properties of ternary complexes of CS and PLA/DNA including complex formation, binding affinity, particle size and zeta potential were characterized because these properties affect the transfection efficiency. PLA (MW >70 kDa) was selected to form complexes with CS/DNA because PLA showed the highest transfection efficiency. The CS/DNA complexes with PLA were prepared by charge interaction induced self-assembly at various weight of PLA ranging from 0 to 50 μg, whereas DNA and CS were fixed at 1 μg and 0.01 μg, respectively. Three types of order of mixing were (1) PLA/DNA/CS; (2) CS/DNA/PLA and (3) PLA/CS/DNA. Fig. 2 illustrates gel electrophoresis of these CS/DNA complexes without polypeptide (Fig. 2a) and three types of mixing (Fig. 2b). For CS/DNA complexes, at pH 6.4 the migration of DNA was completely retarded when the weight ratio was above 0.1. The migration of PLA/DNA/CS, CS/DNA/PLA and PLA/CS/DNA complexes was completely retarded when the weight ratio was above 0.5, 0.5 and 0.1, respectively. These results indicated that the order of mixing affected the formation of complete complexes. This might due to the different in binding affinity.

The particle size and zeta potential of those complexes were elucidated. The CS/DNA complexes with PLA were prepared by three types of order of mixing at various weight of PLA ranging from 0 to 4, whereas, DNA and CS were fixed at 1 μg and 4 μg, respectively. As shown in Table 2, the particle size was dependent on both weight ratio and order of mixing between CS, PLA and DNA. The particle size of all complexes was nanosize (130–400 nm), showing that complete complexes were formed. The zeta-potential was positive and rather constant in the range of 23–40 mV.

The binding affinity of CS or PLA to DNA was studied by ethidium bromide (EtBr) displacement assay. EtBr intercalates between the base pairs of the DNA double helix, yielding a highly fluorescent EtBr/DNA complexes. As polycation was introduced to the DNA, the fluorescence emitted because EtBr/DNA complexes decreased until a minimum level was reached close to baseline fluorescence. The degree of displacement of EtBr by CS or PLA illustrates the binding affinity, indicating the relative strength of the interaction between CS or PLA and DNA. Fig. 3 shows the change in fluores-

Table 1
The particle sizes and zeta-potential of the polypeptides/DNA complexes at pH 6.4 and 7.4.

Weight ratio	PLL/DNA				PLA/DNA				PLO/DNA			
	pH 6.4		pH 7.4		pH 6.4		pH 7.4		pH 6.4		pH 7.4	
	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)
1	362 ± 22	45 ± 1.6	257 ± 18	15 ± 0.6	282 ± 34	27 ± 0.5	267 ± 15	16 ± 0.6	474 ± 38	50 ± 13	557 ± 78	19 ± 1.3
2	541 ± 71	48 ± 5.3	337 ± 39	20 ± 1.3	252 ± 12	35 ± 3.4	317 ± 6	20 ± 0.3	500 ± 20	53 ± 1.1	340 ± 10	22 ± 1.4
3	478 ± 44	57 ± 1.7	377 ± 22	27 ± 0.9	340 ± 23	39 ± 2.4	331 ± 13	26 ± 3.9	283 ± 24	23 ± 2.1	554 ± 24	23 ± 0.7
4	402 ± 36	59 ± 7.1	364 ± 25	27 ± 0.4	342 ± 18	39 ± 0.8	309 ± 46	27 ± 0.6	752 ± 37	52 ± 6.4	134 ± 27	29 ± 3.4

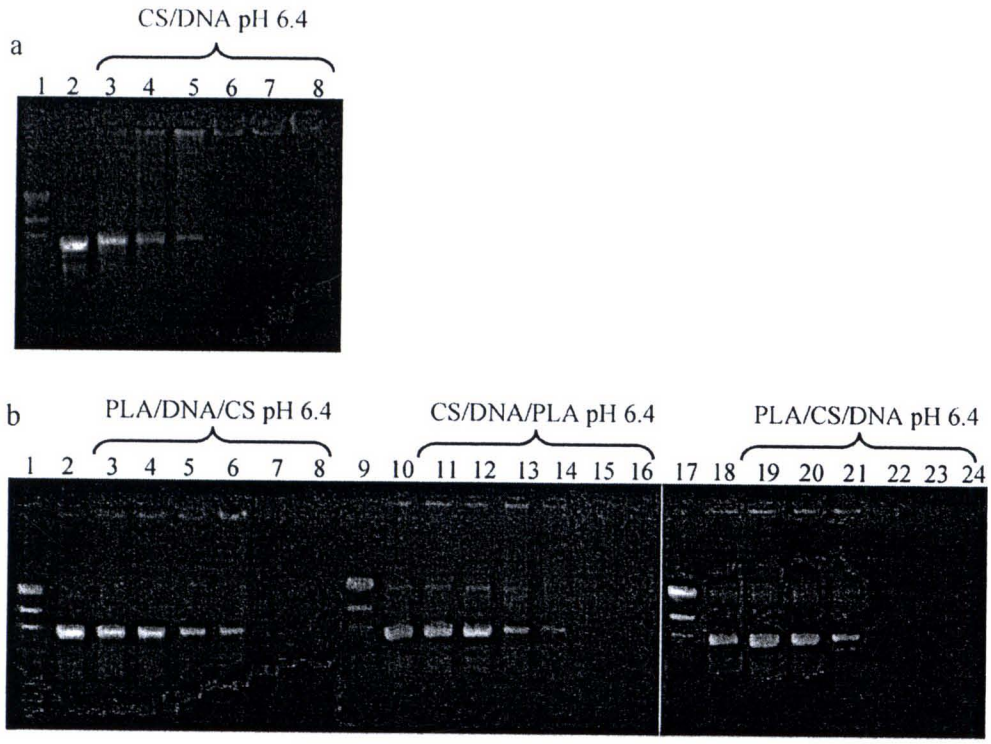

Fig. 2. Gel retarding analysis of (a) CS/DNA complexes at pH 6.4. Lane 1, DNA marker; lane 2, pEGFP-C2 plasmid; lanes 3–8, CS/DNA complexes at weight ratios of 0.01, 0.05, 0.1, 0.05, 1 and 4. (b) CS/DNA at pH 6.4 with increasing weight ratio of PLA and different order of mixing, DNA and CS were fixed at 1 μg and 0.01 μg, respectively. Lanes 1, 9, 17 DNA marker; lanes 2, 10, 18 pEGFP-C2 plasmid; lanes 3–8, 11–16, 19–24 of PLA/DNA complexes at weight ratios of 0.01, 0.05, 0.1, 0.5, 1 and 2, respectively, with three different orders of mixing (PLA/DNA/CS, CS/DNA/PLA and PLA/CS/DNA).

Table 2
The particle sizes and zeta-potential of the ternary complexes at pH 6.4.

Amount of PLA (μg)	PLA/DNA/CS		CS/DNA/PLA		PLA/CS/DNA	
	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)
1	229 ± 8	33 ± 2.3	377 ± 18	39 ± 1.2	398 ± 5.9	39 ± 1.3
2	248 ± 6.2	33 ± 2.8	237 ± 3	39 ± 1.2	353 ± 31	34 ± 1.6
3	175 ± 3.4	31 ± 1.3	269 ± 9	39 ± 0.9	261 ± 7.6	30 ± 1.5
4	231 ± 18	33 ± 0.4	409 ± 30	35 ± 3.4	134 ± 27	29 ± 3.4

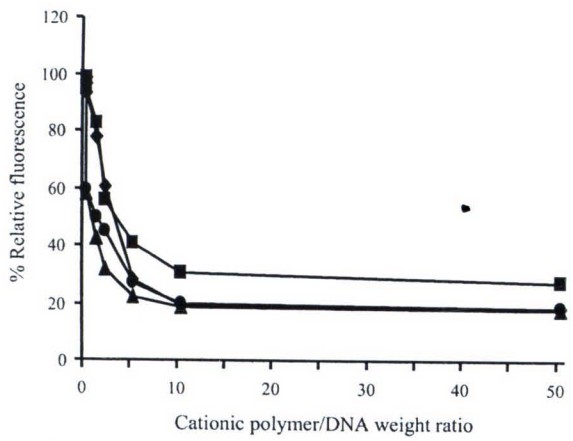

Fig. 3. Percentage relative fluorescent intensity of DNA/EtBr complex containing 10 μg DNA in the complex formulation with increasing amounts of CS and PLA for CS/DNA (■) and PLA/DNA (♦) complexes, respectively. For CS/DNA/PLA (▲) and PLA/DNA/CS (●) complexes CS was fixed at 4 μg with increasing amounts of PLA. Each value represents the mean ± SD of three measurements.

cence intensity of samples relative to a control containing 10 μg DNA in the complex formulation with increasing amounts of CS and PLA for CS/DNA and PLA/DNA complexes, respectively. For CS/DNA/PLA and PLA/DNA/CS complexes, CS was fixed at 4 μg with increasing amount of PLA. The DNA/EtBr complex without titration of CS or PLA was considered as a control with a relative fluorescent intensity of 100%. With the increase in the amount of CS or PLA, the fluorescent intensity showed a decreasing trend, indicating EtBr was replaced by the added CS or PLA, that is, CS or PLA bound selectively to DNA. This could be the increase in the interaction of CS or PLA and DNA when the amount of CS or PLA increased. In addition, the binding affinity reached a plateau at high weight ratio. The rank of binding affinity of the CS or PLA to DNA were PLA/DNA/CS ≈ CS/DNA/PLA > CS/DNA ≈ PLA/DNA. The result is in close agreement with the results previously reported by Liu et al. (2005) and Strand et al. (2005). They found that the binding affinity of chitosan to DNA increased with increasing the N/P ratio. In addition, the binding affinity reached a plateau at high N/P ratio. Our data on ethidium exclusion indicated that the mixture of CS and PLA showed a stronger binding between polycation and DNA than CS or PLA alone.

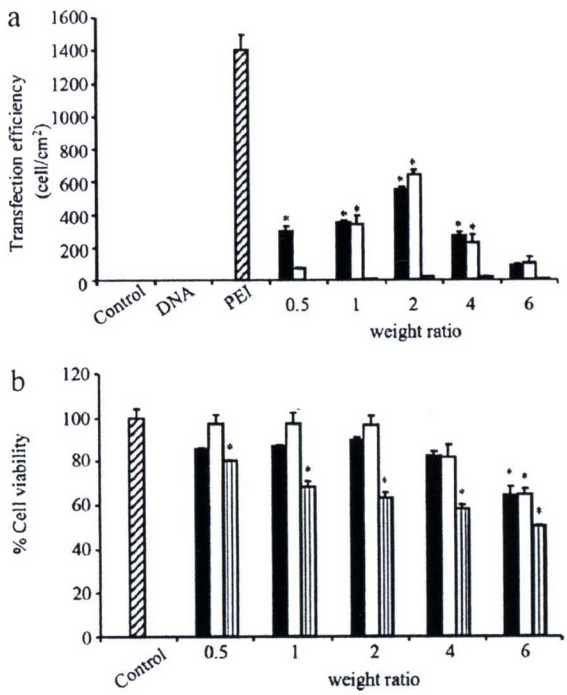

Fig. 4. Effect of weight ratios on (a) transfection efficiency and (b) cell viability of polypeptides/DNA complexes with various weight ratios of 0.5–6 at pH 7.4 in HeLa cells; (■) PLL MW 30–70 kDa, (□) PLA MW 15–70 kDa and (▯) PLO MW 30–70 kDa. Values shown are the means ± SD of triplicate experiments (* indicates $p \leq 0.05$).

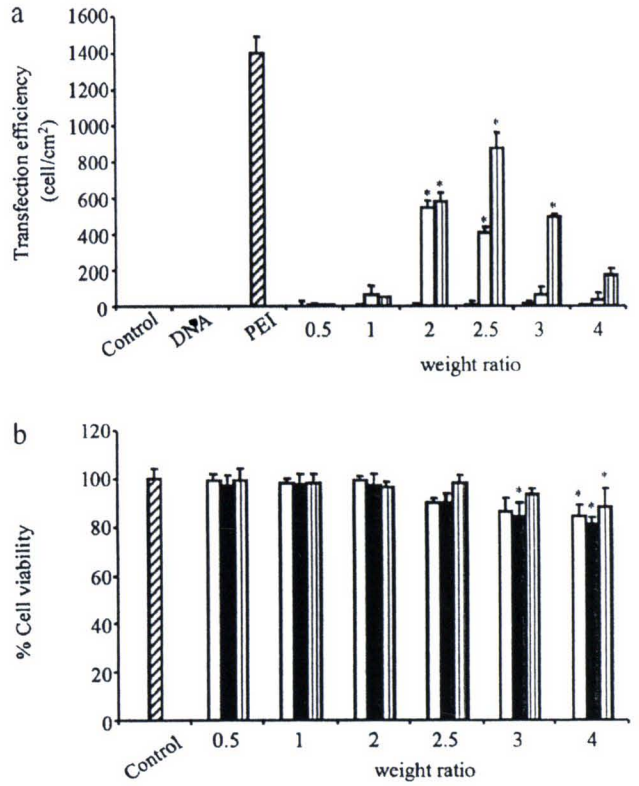

Fig. 5. Effect of molecular weight of PLA on (a) transfection efficiency and (b) cell viability of polypeptides/DNA complexes with various weight ratios of 0.5–6 at pH 6.4 in HeLa cells; (■) PLA MW 5–15 kDa, (□) PLA MW 15–70 kDa and (▯) PLA MW >70 kDa. Values shown are the means ± SD of triplicate experiments (* indicates $p \leq 0.05$).

3.3. *In vitro transfection and cell viability of polypeptide/DNA complexes*

The gene delivery potential of polymer in mammalian cells depends on several factors such as type of polymer, polymer MW, weight ratio and pH of transfection medium/complex solution (Sato et al., 2001). Therefore, transfection conditions such as type and MW of polypeptides, weight ratio and pH of transfection medium were investigated in HeLa cells by transfected-cell counting using images obtained by fluorescence microscope. In the first experiment, we studied the effect of polypeptides type (PLL, PLA and PLO) on transfection efficiency by various weight ratios (0.5, 1, 2, 4, 6) of polypeptides/DNA complexes. Polyethylenimine (PEI, 25 kDa) complexed with DNA at the weight ratio of 1 was used as a positive control. In all studies, there were no transfection in control (cells without complexes) and naked DNA. As shown in Fig. 4a (at pH 7.4), the gene transfection efficiencies were significantly influenced by the carrier/DNA ratios. By increasing the ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. The maximum transfection efficiency achieved at the weight ratio of 2. Among the carriers, PLA showed the highest transfection efficiency. The rank of maximum transfection efficiency of the polypeptides were PLA > PLL > PLO. To test whether pH had an effect on transfection, HeLa cells were transfected with PLL/DNA, PLA/DNA and PLO/DNA complexes at pH 6.4. The results revealed that transfection efficiency of cationic polypeptides at pH 6.4 was not significantly different to that of pH 7.4 (data not shown). The highest transfection efficiency was obtained with PLA/DNA complexes. The viability of HeLa cells was also tested in the presence of polypeptides/DNA complexes at various weight ratios as studied in the transfection experiment (Fig. 4b). The naked DNA did not show any cytotoxicity effect on the cells, and the cell viability was maintained around 100%. The results showed that cationic polypeptides showed a significant decrease in cell viability with increasing in weight ratio of polypeptides/DNA complexes. PLL/DNA and PLA/DNA complexes at weight ratio less than 4, approximately 80% of cells were viable. PLO/DNA complexes had the highest cytotoxicity level and the average cell viability was less than 80% at the weight ratio more than 0.5. The cytotoxicity of cationic polymer was probably caused by polymer aggregation on cell surfaces impairing the important membrane function (Jiang et al., 2007). These results illustrated that there is the extreme importance of polypeptide type to gene transfer mediated, which is a unique property of this vector. Therefore, the PLA/DNA complexes were the condition chosen for further exploring experiments.

In order to investigate the effect of MW of polypeptides on the transfection efficiency and on the cell viability, PLA with different MW (5–15, 15–70 and >70 kDa) was used and the results of the transfection efficiencies and the cell viability are shown in Fig. 5a and b, respectively. As the weight ratio increased, the transfection efficiency had a tendency to increase, whereas cell viability significantly decreased. However, there was no significant difference in the cell viability in the different MW of PLA. At different MW of PLA, the maximum transfection efficiency was found at different weight ratio. PLA/DNA complexes of MW 5–15, 15–70 and >70 kDa showed maximum transfection efficiency at the weight ratios of 3 (17 ± 3 cell/cm^2), 2 (545 ± 36 cell/cm^2) and 2.5 (872 ± 40 cell/cm^2), respectively. MW >70 kDa showed the highest transfection efficiency, whereas, the cell viability was not significant different. Thus, the MW of PLA in the range studied affected the transfection efficiency. The high transfection efficiency of high MW cationic polymer might be attributed to the highly positive charge complexes that be able to be uptaken by the cells (Zhao et al., 2006). Kim et al. (2003a,b) reported that the efficiency of arginine 15 residues is almost sevenfold higher than that of commercial transfection agents (Lipofectin)

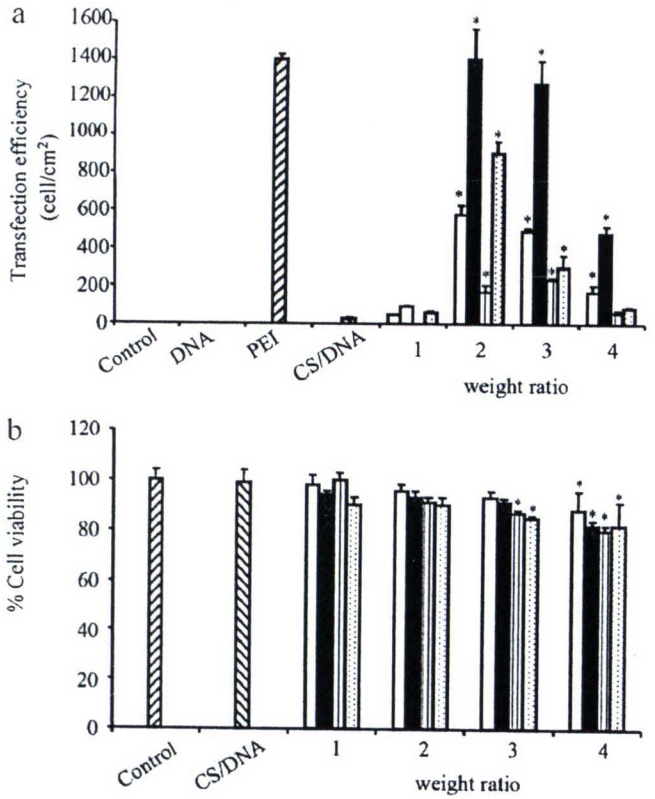

Fig. 6. Effect of order of mixing on (a) transfection efficiencies (b) cell viability of CS/DNA (4:1) complexes at varying weight ratios of PLA with three different order of mixing; at pH 6.4 in HeLa cells; (□) PLA/DNA, (■) PLA/DNA/CS, (◨) CS/DNA/PLA, and (◫) PLA/CS/DNA. Each value represents the mean ± SD of three wells (* indicates $p \leq 0.05$).

and arginine 12 and 9 residues in 293T cells. The main drawback of these low MW peptide–DNA formulations is their instability during in vitro and in vivo gene delivery because the poor peptide–DNA binding leads to easy DNA release.

3.4. In vitro transfection and cell viability of CS and PLA/DNA complexes

Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 and the maximum transfection efficiency of the CS/DNA complex achieved at the weight ratio of 4 (Weecharangsan et al., 2008). Therefore, in this study CS and DNA were fixed at 4 μg and 1 μg, respectively, with various weight ratios of PLA MW >70 kDa and order of mixing. Three types of order of mixing used in this study were (1) PLA/DNA/CS, (2) CS/DNA/PLA and (3) PLA/CS/DNA. The results of the transfection efficiencies and the cell viability are shown in Fig. 6a and b, respectively. The mixing of two polymers (CS and PLA) complexed with DNA showed higher transfection efficiency than that of CS/DNA complexes (weight ratio of 4 about 30 ± 4 cell/cm^2) or PLA/DNA complexes (weight ratio of 2 about 578 ± 34 cell/cm^2). The transfection efficiency was dramatically depended on the order of mixing of these CS, PLA and DNA. All types of complexes had a tendency to increase the transfection efficiency as the weight ratio of PLA increased (Fig. 6a). At the low weight ratio of 1, the transfection efficiency of all complexes was not different in comparing with CS/DNA. This might be resulted from the insufficient amount of PLA. Among the order of mixing, PLA/DNA/CS (2:1:4) showed the highest transfection

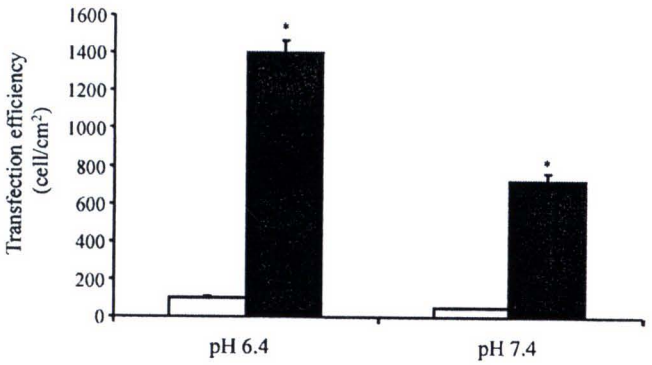

Fig. 7. Effect of pH medium at pH 7.4 and pH 6.4 with 10% serum (□) and without serum (■), on transfection efficiencies of PLA/DNA/CS complexes at the weight ratio of 2:1:4 in HeLa cells. Each value represents the mean ± SD of three wells (* indicates $p \leq 0.05$).

efficiency (1407 ± 155 cell/cm^2). Its transfection efficiency was 6 and 1.6 times higher than that of CS/DNA/PLA (4:1:3; 230 ± 12 cell/cm^2) and PLA/CS/DNA (2:4:1; 900 ± 66 cell/cm^2), respectively. The transfection efficiency of PLA/DNA/CS complexes was not significantly different in comparing with that of PEI/DNA complex at weight ratio of 1 (1400 ± 28 cell/cm^2). On the other hand, it was significantly higher than that of PLA/DNA complexes (2.4 times) and CS/DNA complexes (47 times), respectively. The transfection efficiency of carriers could be ranked as follows: PLA/DNA/CS > PLA/CS/DNA > PLA/DNA > CS/DNA/PLA > CS/DNA.

Our results clearly demonstrate that order of mixing in ternary complexes, resulting from the association of CS to PLA/DNA complexes, are significantly more efficient in mediating transfection than other complexes. Although the exact mechanism of CS mediated efficient gene delivery of PLA/DNA complexes remains to be further studied, our preliminary study in determining the uptake pathways of those complexes using endocytosis inhibitors showed that entry of PLA/DNA/CS complexes may differ from that of PLA/DNA complexes and CS/DNA complexes. Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 (Weecharangsan et al., 2008). As shown in Fig. 7, pH dramatically affected CS with PLA with decreasing the transfection efficiency by increasing pH values from 6.4 to 7.4. In addition, 10% serum containing media significantly decreased the transfection efficiency both in pH 6.4 and 7.4. Although the exact mechanism of CS to PLA/DNA complexes mediated efficient gene delivery remains to be further studied, our results clearly demonstrate that the addition of CS to PLA/DNA could thereby influence the physical properties of the heteroplex such as particle size, surface charge distribution, DNA condensation, and intracellular stability. These effects could influence the transfection efficiency of the complex and moreover, could be potential candidate for non-viral gene carriers. The ternary complexes displayed low cytotoxicity (Fig. 6b) and had lower cytotoxicity than the complex of PEI 25 kDa. There was a slight decrease in cell viability when HeLa cells were incubated with increasing amount of PLA and the cell viability was over 80% in all order of mixing complexes. This might be the result of biodegradable property of polypeptide and CS that leads to lower cytotoxicity (Wong et al., 2006).

4. Conclusion

Our results clearly demonstrate that ternary complexes, resulting from association of CS to PLA/DNA complexes, are significantly more efficient in mediating transfection than the corresponding

PLA/DNA or CS/DNA complexes and had low cytotoxicity. Our findings suggest that at optimal ratio the high gene expression can be achieved by adding CS to PLA/DNA complexes without a covalent conjugation.

Acknowledgments

The authors would like to acknowledge the financial support from Silpakorn University Research and Development Institute (SURDI 53/01/19), Commission of Higher Education (Thailand) and the Thailand Research Fund (Project No. DBG5180005) and National Nanotechnology Center (NANOTEC), Thailand (grant number: NN-B-22-EN3-17-52-10).

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Please cite this article in press as: Opanasopit, P., et al., Chitosan enhances transfection efficiency of cationic polypeptides/DNA complexes. Int. J. Pharm. (2011), doi:10.1016/j.ijpharm.2011.03.008

International Journal of Nanomedicine

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ORIGINAL RESEARCH

Type and composition of surfactants mediating gene transfection of polyethylenimine-coated liposomes

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Background: The objective of this study was to compare the transfection efficiency of anionic liposomes coated with polyethylenimine (PEI) with that of PEI and Lipofectamine 2000™ using the plasmid DNA encoding green fluorescent protein in a human hepatoma (Huh7) cell line.

Methods: Factors affecting transfection efficiency, including type of surfactant, ratio of phosphatidylcholine (PC)/surfactant, carrier/DNA weight ratio, and the presence of serum have been investigated. Anionic liposomes, composed of PC and anionic surfactants, ie, sodium oleate (NaO), sodium taurocholate (NaT), or zwitterionic surfactant (3-[{3-cholamidopropyl}-dimethylammonio]-1-propanesulfonate, CHAPS) at molar ratios of 10:1, 10:1.5, and 10:2 were prepared by the sonication method. Subsequently, they were coated with PEI to produce polycationic liposomes (PCL).

Results: PCL was able to condense with pDNA depending on the PCL/DNA weight ratio. PCL composed of PC:NaO (10:2) showed higher transfection efficiency than NaT and CHAPS at all weight ratios tested. Higher transfection efficiency and gene expression were observed when the carrier/DNA weight ratio increased. The highest transfection efficiency was found at a weight ratio of 0.5.

Conclusion: This PCL showed remarkably high transfection efficiency with low cytotoxicity to Huh7 cells in vitro, in comparison with PEI and Lipofectamine 2000.

Keywords: polycation liposomes, polyethylenimine, gene delivery, transfection efficiency

Introduction

Gene delivery has been regarded as a powerful tool for curing disease by replacing defective genes, substituting missing genes, or silencing unwanted gene expression. The two main types of vectors used in gene therapy are viral and nonviral vectors. Among the nonviral vectors, cationic liposomes[1–3] and polycations[4,5] have been widely investigated. Cationic liposomes form a complex with anionic DNA molecules and are thought to deliver DNA through the endosomal pathway.[6] The precise mechanism for gene transfection mediated by cationic liposomes is still unclear. However, fusion with the endosomal membrane or destabilization of the membrane by cationic liposomes may trigger the effective cytosolic delivery of DNA.[7,8] However, the cationic liposomal system has some disadvantages, such as low efficiency of transfection, due to DNA degradation in lysosomes, and strong cytotoxicity.[9] On the other hand, polycations, such as polyethylenimine (PEI), also form a complex with anionic DNA molecules and are taken up into target cells via the endosomal pathway. However, in the case of polycations, unlike cationic liposomes, the DNA released into the cytoplasm from

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endosomes, due to the proton-sponge effect of PEI, may further be delivered to the nucleus by the polycation.[10,11]

Yamazaki et al[12,13] prepared polycation liposomes (PCL) by modification of a liposome with cetyl PEI, and found that PCL could actually deliver genes effectively in the presence of serum and in vivo with low cytotoxicity. The mechanism for effective gene transfer by PCL was that PCL transfer DNA to endosomes and release cetyl PEI/DNA complexes into the cytosol. Furthermore, cetyl PEI also contributes to gene entry into the nucleus.[14] Chen et al[15] also prepared PCL using PEI (molecular weight 800) and cholesterol. These PCL had transfection efficiency equivalent to that of Lipofectamine 2000™ with significantly low cytotoxicity. In general, the key step of gene delivery is thought to be not the delivery of DNA into the cytosol through the endosomal pathway but rather the entry of cytosolic DNA into the nucleus when cationic liposomes are used as a nonviral vector.[16] Therefore, gene delivery is cell-cycle dependent, and effective gene delivery is achieved only in dividing cells. If PEI has the ability to enter into the nucleus in nondividing cells, then PCL may have the dual advantage of both cationic liposomes for cytosolic delivery of DNA and polycations for delivery of cytosolic DNA into the nucleus. Alternatively, if PEI cannot pass through the nuclear membrane, it still has the ability to enhance interaction of foreign DNA with chromatins during cell division.

Although many researchers have attempted to develop an effective nonviral gene transfer system by combination of both cationic liposomes and polycations as an alternative gene carrier, few have been successful in increasing transfection efficiency. Lampela et al have reported that the use of PEI cationic liposomes (Dosper) for nonviral gene delivery showed synergism between cationic lipid Dosper and small molecular weight PEI in transfection.[17] They suggested that this synergism was based on two independent mechanisms, ie, the liposome improves cellular uptake of PEI/DNA complexes, and PEI improves the transfer of the complexes from the lysosomes to the nucleus. They found that high molecular weight PEI with linear (PEI 22 K) and branched (PEI 25 K) structures in combination with Dosper liposomes could be used to increase the efficiency of gene delivery in vitro.[18] Moreover, they combined low molecular weight PEI (MW 700 and 2000) with Superfect (a dendrimer) and two liposomes, ie, Dotap (N-[1-{2,3-dioleoyloxy}propyl]-N,N,N-trimethylammonium methylsulfate) and Lipofectamine. The highest synergism was achieved with Lipofectamine and PEI in Rabbit smooth muscle (SMC) cells, or with Dotap and PEIs in rat glioma (C6) cells. Superfect did not induce any synergism.[19] Therefore, in this study, PEI-coated liposomes with different compositions were prepared to investigate the physicochemical properties intended for gene delivery. Surfactants, ie, (3-[{3-Cholamidopropyl} dimethylammonio]-1-propanesulfonate, CHAPS), sodium taurocholate (NaT), or sodium oleate (NaO) was added to phosphatidylcholine (PC) to form an anionic liposome. A number of variables influencing transfection efficiency, such as carrier/DNA weight ratio, type of surfactants (CHAPS, NaT and NaO), particle size, zeta potential, morphology, and serum were investigated. In vitro gene transfection assay was performed with a human hepatoma (Huh7) cell line using plasmid DNA encoding green fluorescent protein (pEGFP-C2). Evaluation of cytotoxicity was performed by the MTT assay. The results revealed that PEI-coated anionic liposomes PC:NaO (10:2) showed high transfection efficiency with low cytotoxicity to Huh7 cells in vitro, in comparison with PEI and Lipofectamine 2000.

Material and methods

Materials

Egg yolk PC was purchased from Wako Pure Chemical (Osaka, Japan). Branched PEI, (molecular weight 25 kDa, 3-[{3-Cholamidopropyl}dimethyl ammonio]-1-propanesulfonate, CHAPS), sodium oleate (NaO), and sodium taurocholate (NaT), Tris, Triton-X 100, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), trypsin-ethylenediamine tetra-acetic acid (EDTA), penicillin-streptomycin antibiotics, fetal bovine serum, and Lipofectamine 2000 were obtained from Gibco Invitrogen (Grand Island, NY). pEGFP-C2 was obtained from Clontech (Palo Alto, CA). The λDNA/HindIII marker was obtained from Promega (Madison, WI). Human hepatocellular carcinoma (Huh7) cells were obtained from the American Type Culture Collection (Rockville, MD). All other chemicals were of cell culture and molecular biology quality.

Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA). DNA concentration was quantified by the measurement of ultraviolet absorbance at 260 nm using a GeneRay ultraviolet photometer (Biometra®, Goettingen, Germany). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate-EDTA buffer, pH 8.0, and using λDNA/HindIII as a DNA marker.

Preparation of PEI-coated liposomes

Different formulations composed of a bilayer forming PC, in combination of NaO, CHAPS, or NaT, in molar ratios of 10:1, 10:1.5, and 10:2, were prepared by the sonication method. Briefly, PC, CHAPS, NaO, and NaT were separately dissolved in chloroform:methanol (2:1 v/v). The materials were deposited in a test tube and the solvents were evaporated under nitrogen gas flow. The lipid film was placed in a desiccator connected to a vacuum pump for six hours to remove remaining organic solvents. The dried lipid film was hydrated with Tris buffer (20 mM Tris and 150 mM NaCl, pH 7.4). Following hydration, the dispersion was sonicated in a bath sonicator for 10 minutes and then in a probe sonicator, each for 30 minutes in two cycles. For PEI-coated liposomes, the liposomes were mixed with PEI solution (1 mg/mL) at the ratio of 1:1 (w/w) with a magnetic stirrer for 30 minutes.

Preparation and characterization of PCL/DNA complexes

The PCL/DNA complexes were prepared at various carrier/DNA weight ratios by adding DNA solution to the PCL solution. The mixture was gently mixed using a pipette for 3–5 seconds to initiate complex formation and was left for 15 minutes at room temperature. The complex formation was confirmed by electrophoresis. Agarose gels were prepared with 1% agarose solution in Tris acetate-EDTA buffer with ethidium bromide 0.5 μg/mL. The electrophoresis was carried out for 60 minutes at 100 V. The volume of the sample loaded in the well was 15 μL of PCL/DNA complex containing 1 μg of DNA.

Size and zeta potential measurements

The particle size and surface charge of the PCL/DNA complexes were determined by photon correlation spectroscopy using the Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) at room temperature. The complexes were diluted with distilled water which was previously passed through a 0.22 μm membrane filter. All samples were measured in triplicate.

Morphology

The morphology of the PCL/DNA complexes was analyzed by transmission electron microscopy and atomic force microscopy. Analyzed by transmission electron microscopy, 3% solution of Formvar was prepared in spectroscopic-grade chloroform. Then, one drop of the sample solution of PCL/DNA complex was put on a Formvar-coated carbon ultrathin grid and air-dried. The dried grid was then examined by transmission electron microscopy (JEOL JEM1230, Tokyo, Japan). Analyzed by atomic force microscopy (SPA400, Seiko, Japan), appropriate amounts of PCL/DNA complexes were diluted with water and deposited onto a freshly cleaved mica substrate. Samples were imaged after evaporation to dryness by scanning a 5000 nm × 1000 nm area in tapping mode using a NSG 10 cantilever with 190–325 kHz resonance frequencies and a constant force in the range of 5.5–22.5 N/m. The images were recorded in air at room temperature and a scan speed of 1 Hz, and the phase image and topology were used to determine the morphology and particle size of the liposomes.

In vitro transfection PCL/DNA complexes in Huh7 cells

Huh7 cells were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 mL of growth medium (DMEM containing 10% fetal bovine serum, supplemented with 2 mM L-glutamine, 1% nonessential amino acid solution, 100 U/mL penicillin, and 100 μg/mL streptomycin). The cells were grown in a humidified atmosphere (5% CO_2, 95% air, 37°C) for 24 hours. Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (pH 7.4). The cells were incubated with 0.5 mL of the PCL/DNA complexes at various weight ratios containing 1 μg of pDNA for 24 hours at 37°C in a 5% CO_2 atmosphere. Nontreated cells and cells transfected with naked plasmid, PEI/DNA complexes, and Lipofectamine 2000/DNA complexes were used as controls. After transfection, the cells were washed twice with phosphate-buffered saline and grown in culture medium for 24 hours to allow for green fluorescent protein expression. All transfection experiments were performed in triplicate.

Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. Huh7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μL of growth medium and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Prior to evaluation of cytotoxicity, the medium was removed and the cells were rinsed with phosphate-buffered saline. They were supplied with the PCL/DNA complexes in the same concentrations as for the in vitro transfection experiment. After treatment, the PCL/DNA complex solutions were removed. Finally, the cells were incubated with 100 μL MTT-containing medium (1 mg/mL) for four hours. Then the medium was removed, the cells were rinsed with phosphate-buffered saline (pH 7.4), and formazan crystals formed in

living cells were dissolved in 100 μL dimethyl sulfoxide per well. Relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Models AOPUS01 and AI53601, Packard BioScience, East Lyme, CT). The viability of non-treated control cells was arbitrarily defined as 100%.

Statistical analysis

The statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance followed by a least-squares difference post hoc test. The significance level was set at $P < 0.05$.

Results and discussion

Characterization of PCL/DNA complexes

In order to determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between either PEI or PCL and DNA at different carrier concentrations. The formation of complexes between PCL and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. By varying the concentration of PCL and fixing the DNA concentration, the weight ratios of the particle formulations were varied (Figure 1; Lane 1, pEGFP-C2 plasmid; lanes 2–7, PCL/DNA complexes with weight ratios of 0.05, 0.1, 0.5, 1, 5, and 10). As shown in Figure 1, PCL/DNA complexes showed different gene condensation patterns. In the case of PC/NaO liposomes (Figure 1A–C), complete complexes were formed at carrier/DNA weight ratios above 0.05 in all PC/NaO ratios (10:1, 10:1.5, and 10:2). On the other hand, PC/CHAPS (Figure 1D–F) and PC/NaT (Figure 1G–I) formed complete complexes with DNA at carrier/DNA weight ratios above 0.1, 0.05, and 0.05 for PC/CHAPS and PC/NaT at ratios of 10:1, 10:1.5, and 10.2, respectively. These results revealed that the NaO had a stronger binding ability to DNA than that of CHAPS and NaT.

Particle size and the zeta potential were plotted against weight ratios of PEI/DNA complexes (Figure 2A) and PCL/DNA complexes formulated with PEI-coated PC/NaO (10:2) liposomes (Figure 2B), PEI-coated PC/CHAPS (10:2) liposomes (Figure 2C), and PEI-coated PC/NaT (10:2) liposomes (Figure 2D). The particle sizes of the PEI/DNA complexes were in the 200–500 nm range (PDI 0.21–0.64) after a weight ratio of 0.75 (Figure 2A). The particle sizes of PLC formulated from PC/NaO and PC/CHAPS liposomes were slightly larger than the PEI/DNA complexes, whereas PLC formulated from PC/NaT showed the largest particle size (550–1595 nm). An initial negative value of the zeta potential was observed for all anionic liposomes (PC/NaO, PC/CHAPS, and PC/NaT). On the other hand, PEI-coated liposomes at 1:1 (w/w) showed positive values for the zeta potential. The zeta potential of all the PCL/DNA complexes was found to increase with increasing weight ratios of PCL, due to their higher density of protonated amines in the PCL. A similar result was observed in PEI (Figure 2A). Morphological examination of the PEI-coated PC/NaO (10:2) liposome/DNA complexes at a weight ratio of 0.5 were performed by transmission electron microscopy and atomic force microscopy. The images revealed that the complexes were spherical and nanosized (Figure 3).

In vitro transfection

Achievement of high gene transfection efficiency is the final goal for the development of novel gene carriers. To investigate PCL-mediated gene transfection efficiency, an in vitro gene transfection assay was performed with a human hepatoma (Huh7) cell line using pEGFP-C2. PCL formulated with three kinds of anionic liposomes (PC/NaO, PC/CHAPS, and PC/NaT at molar ratios of 10:1, 10:1.5, and 10:2, respectively) and then coated with PEI at a weight ratio of 1:1. In order to investigate the optimal conditions for gene transfection, the PCL were complexed with DNA at various weight ratios of 0.3, 0.4, 0.5, 0.75, 0.9, 1.0, and 1.5. PEI and Lipofectamine 2000 complexed with DNA at weight ratios of 1 and 2, respectively, were used as a positive control. In all studies, there were no transfection in controls (cells without complexes) and naked DNA. As shown in Figure 4 (at pH 7.4), the gene transfection efficiencies were significantly influenced by the carrier/DNA ratio and the type and ratio of surfactants used in the liposome formulation. For PC/NaO (Figure 4A–C) and PC/CHAPS (Figure 4D–F), by increasing the molar ratio of surfactant (from 10:1 to 10:2), the transfection efficiencies increased, and the highest values were observed at the molar ratio of 10:2, whereas the highest values of transfection efficiencies of PC/NaT (Figure 4G–I) were at the molar ratio of 10:1.5. In all PCL, the gene transfection efficiencies were significantly influenced by the carrier/DNA ratio. By increasing the ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. Among the carriers, PEI-coated PC/NaO (10:2) showed the highest transfection efficiency (Figure 4C). Some PCL formulations were much more effective in gene transfer than conventional polyplexes of PEI. PCL containing PC/NaO (10:2, carrier/DNA weight ratio 0.5) showed five times higher gene expression than the polyplex prepared with the same polymer of PEI (Figure 5A). These results suggest that PCL may have the dual advantages of being both liposomes for

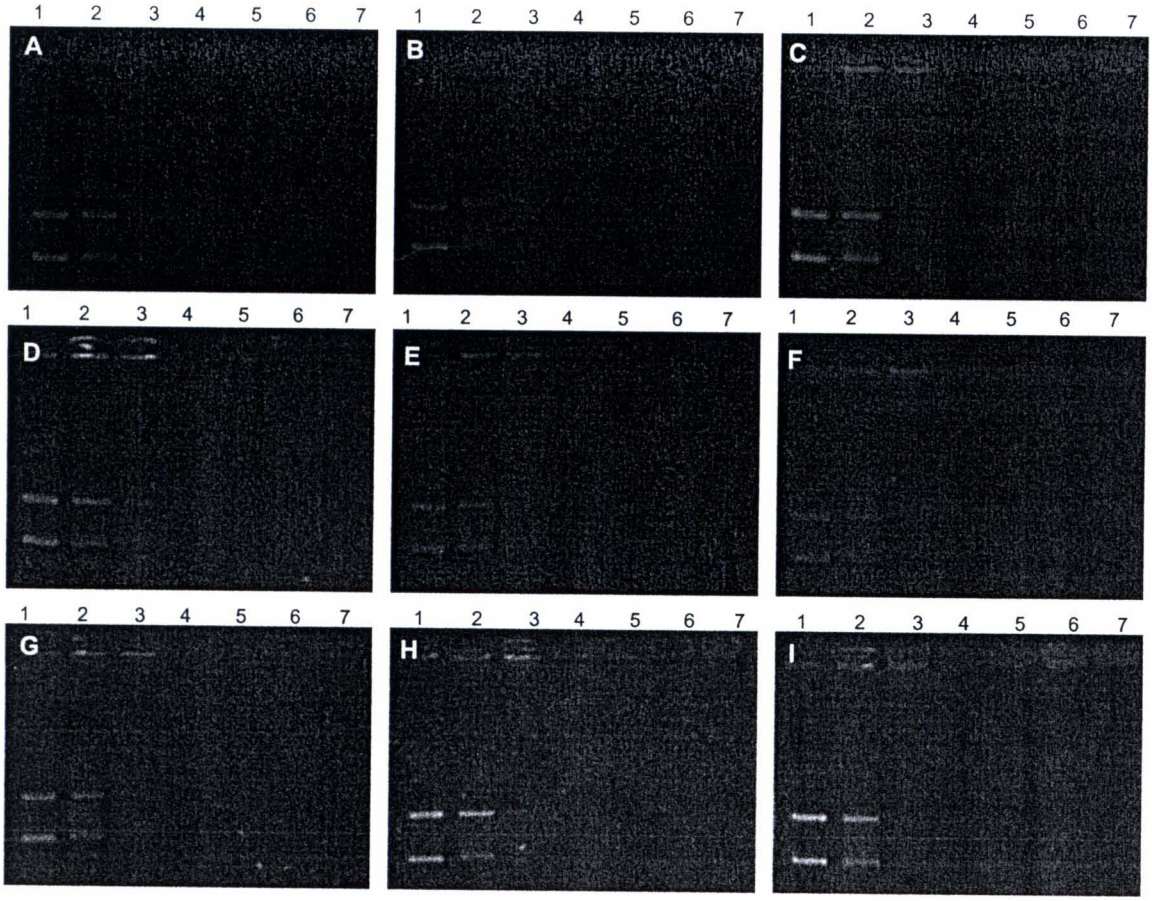

Figure 1 Gel retarding analysis of PCL/DNA complexes formulated with **A)** PC/NaO (10:1), **B)** PC/NaO (10:1.5), **C)** PC/NaO (10:2), **D)** PC/CHAPS (10:1), **E)** PC/CHAPS (10:1.5), **F)** PC/CHAPS (10:2), **G)** PC/NaT (10:1), **H)** PC/NaT (10:1.5), and **I)** PC/NaT (10:2). Lane 1, pEGFP-C2; lanes 2–7, PEI-coated liposomes (PCL)/DNA complexes at weight ratios of 0.05, 0.1, 0.5, 1, 5, and 10, respectively.

Abbreviations: PC, phosphatidylcholine; PCL, polycationic liposomes; PEI, polyethylenimine; NaO, sodium oleate; NaT, sodium taurocholate; CHAPS (3-[{3-cholamidopropyl}-dimethylammonio]-1-propanesulfonate) (zwitterionic surfactant); pEGFP-C2, plasmid DNA encoding green fluorescent protein.

cytosolic delivery of DNA and PEI for delivery of cytosolic DNA into the nucleus if PEI has the ability to enter into the nucleus in nondividing cells. Alternatively, if PEI cannot pass through the nuclear membrane, PEI still has ability to enhance the interaction of foreign DNA with chromatins during cell division.[16,17]

One of the major requirements for vectors used for gene delivery is low cytotoxicity. Therefore, the cytotoxicity study of the PCL/DNA complex was performed in Huh7 cells. Figure 5B shows the effect of carrier/DNA weight ratios (0.3, 0.4, 0.5, 0.75, 0.9, 1.0, and 1.5) on cell viability. When Huh7 cells were incubated with 1 μg of naked DNA, cell viability remained almost the same as that seen in nontransfected control cells (data not shown). There was a significant decrease in cell viability when Huh7 cells were incubated with various weight ratios of both PEI/DNA and PCL/DNA complexes. Their average cell viability was decreased when the carrier/DNA weight ratio increased. However, viability was over 80% for the PCL/DNA complexes. Therefore, in this study, PCL were clearly demonstrated to be safe. This result is similar to that of a previous study by Hu et al[20] who developed an efficient gene delivery system by combining PEI/DNA complexes, anionic liposomes, and a streptavidin-monoclonal antibody. They showed that these lipopolyplexes helped to decrease cytotoxicity. Their in vitro experiment in SMMC-7721 cells showed that lipopolyplexes did not have significant toxicity. In contrast, PEI/DNA markedly reduced cell viability.

Previous studies have reported that the transfection efficiency of lipoplexes and polyplex-associated gene expression was inhibited by serum. Serum was shown to inhibit transfection activity depending on the charge and composition of the complexes. It is already known that anionic compounds present in serum often complex with positively charged

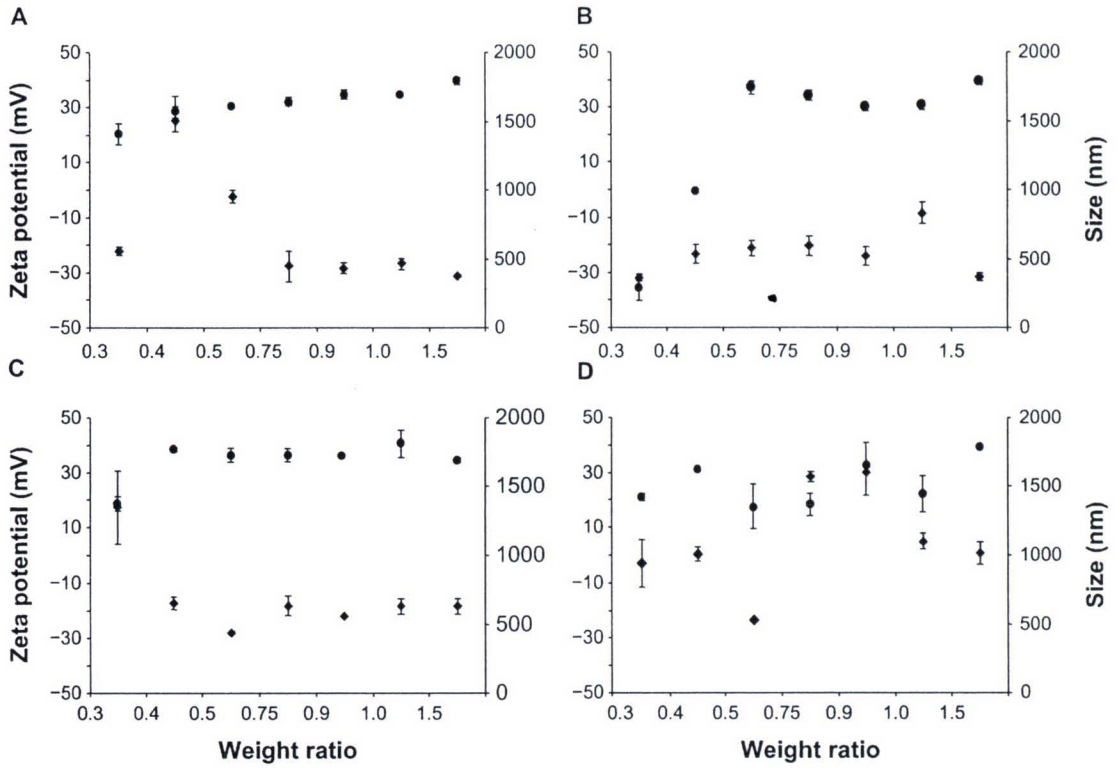

Figure 2 Zeta potential (●) and particle size (◆) at varying weight ratios of PCL/DNA complexes formulated with **A**) PEI, **B**) PEI-coated PC/NaO (10:2) liposomes, **C**) PEI-coated PC/CHAPS (10:2) liposomes, and **D**) PEI-coated PC/NaT (10:2) liposomes.

Note: Each value represents the mean ± standard deviation of three measurements.

Abbreviations: PC, phosphatidylcholine; PCL, polycationic liposomes; PEI, polyethylenimine; NaO, sodium oleate; NaT, sodium taurocholate; CHAPS (3-[{3-cholamidopropyl}-dimethylammonio]-1-propanesulfonate) (zwitterionic surfactant).

transfection reagents, resulting in decreased transfection efficiency.[21,22] These interactions result either in the dissociation of the complexes or in the formation of large aggregates, which, above a certain size limit, may be prevented from being internalized by cells.[23]

As shown in Figure 6, the effect of serum on transfection was dependent on the formulation. Association of gene expression in PCL formulated with three kinds of anionic liposomes, ie, PC/NaO (10:2), PC/CHAPS (10:2), and PC/NaT (10:1.5), was observed in both the presence and absence of serum. For these experiments, the presence of 10% serum in the transfection medium of pH 7.4 decreased the transfection efficiency of PCL formulated with PC/NaO (10:2) and PC/NaT (10:1.5), while that of PCL formulated with PC/CHAPS (10:2) was not significantly affected by the presence of serum. In this regard, it is reasonable to assume that the significant decrease in transfection activity mediated by complexes prepared from PC/NaO (10:2) and PC/NaT (10:1.5) in the presence of serum resulted from interactions with serum proteins. In the case of PC/CHAPS (10:2), CHAPS is a cholic acid derivative in which the carboxylic acid has been amidated with the zwitterionic aminosulfonic acid moiety. Thus, it retains the same hydrophobic-hydrophilic topology as the rest of the bile salt series except that they are formally neutral. CHAPS have gained widespread use in the solubilization and reconstitution of proteins. The solubilization of membrane proteins is affected by the ionic characteristics and critical micellar concentration of detergents. For the membrane proteins, zwitterionic detergents were less efficient

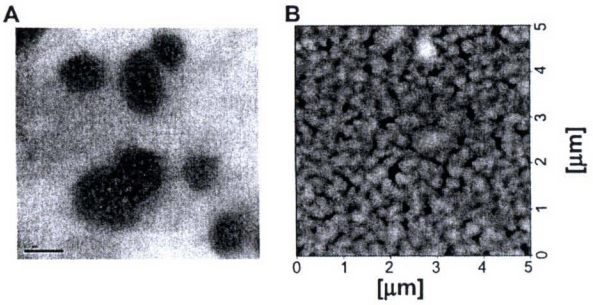

Figure 3 **A**) Transmission electron microscopic (5000×) images, **B**) atomic force microscopic images of PEI-coated PC/NaO (10:2) liposomes/DNA complexes at a weight ratio of 0.5.

Abbreviations: PC, phosphatidylcholine; PEI, polyethylenimine; NaO, sodium oleate.

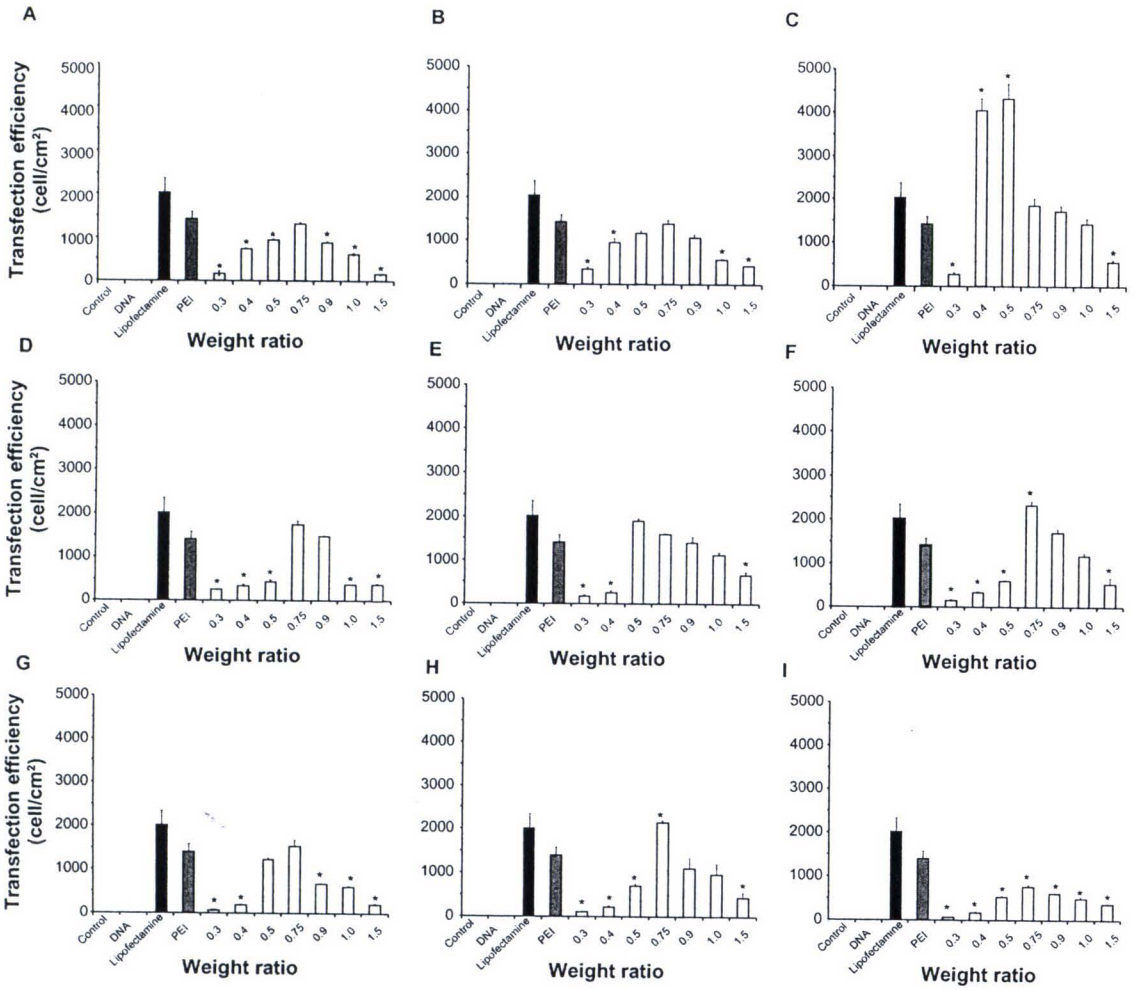

Figure 4 Transfection efficiencies of PCL/DNA complexes formulated with **A)** PC/NaO (10:1), **B)** PC/NaO (10:1.5), **C)** PC/NaO (10:2), **D)** PC/CHAPS (10:1), **E)** PC/CHAPS (10:1.5), **F)** PC/CHAPS (10:2), **G)** PC/NaT (10:1), **H)** PC/NaT (10:1.5), and **I)** PC/NaT (10:2) in Huh7 cells.

Notes: Each value represents the mean ± standard deviation of three wells. * statistically significant ($P < 0.05$).

Abbreviations: PC, phosphatidylcholine; PCL, polycationic liposomes; PEI, polyethylenimine; NaO, sodium oleate; NaT, sodium taurocholate; CHAPS (3-[{3-cholamidopropyl}-dimethylammonio]-1-propanesulfonate) (zwitterionic surfactant).

than ionic detergents.[24,25] Thus, ionic detergents facilitated the incorporation of serum proteins. This observation is consistent with previous reports showing that the formulations containing cholesterol were more active in vivo than those containing dioleoyl phosphatidylethanolamine (DOPE), which has been attributed to their higher stability in biological fluids. This higher stability in the presence of serum is most likely due to the ability of cholesterol to confer membrane stability in contrast with DOPE, which is known to have an inverted cone shape that is associated with membrane fluidity, thus facilitating binding and/or incorporation of serum proteins.[26] Oku et al[13] also reported that, in the presence of serum PCL composed of PC and cetylated PEI (P18C24), there was more enhanced gene transfer than without serum. On the contrary, the efficiency mediated by Dotap liposomes or Lipofectamine was markedly suppressed in the presence of serum. To clarify the reason for serum activation of PCL-mediated transfection, they examined the formation of DNA-PCL complexes under the microscope. PCL and DNA appeared as rather heterogeneous aggregates in the absence of serum, but formed smaller and rather homogeneous ones in the presence of serum. In addition, Marchini et al showed that when serum was added, the transfection efficiency of all lipoplex formulations (didecyldimethylammonium bromide-dioleoylphosphatidylethanolamine and 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol-dioleoylphosphatidylethanolamine) was found to increase. They identified structural stability and an increase in size in serum as major parameters regulating the efficiency of lipofection. By extrapolation, they proposed that serum,

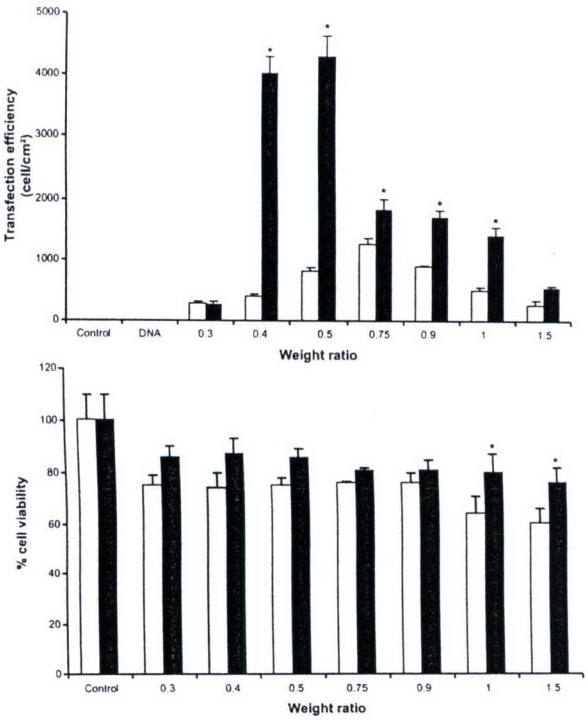

Figure 5 Comparison of the **A)** transfection efficiencies, **B)** cell viability of PEI/DNA (□) and PEI-coated PC/NaO/DNA (■) complexes at various weight ratios in Huh7 cells.

Notes: Each value represents the mean ± standard deviation of three wells. * statistically significant ($P < 0.05$).

Abbreviations: PC, phosphatidylcholine; PEI, polyethylenimine; NaO, sodium oleate.

regulating the size of resistant lipid-DNA complexes, can control the mechanism of internalization of lipoplexes and, in turn, their efficiency.[27] Although conflicting reports exist regarding the optimal size of lipoplexes for lipofection, there is no doubt that high lipofection would be gained from large lipoplexes when endocytosis is dominant, because large particles facilitate membrane contact and fusion. When the type of cell is not an actively endocytosing cell, either small particles may have high transfection efficiency, or lipoplex size may not correlate with lipofection efficiency. In addition to lipoplex size, different conditions (such as cationic lipids, cell types and in vivo/in vitro) may result in different transfection efficiency.[28]

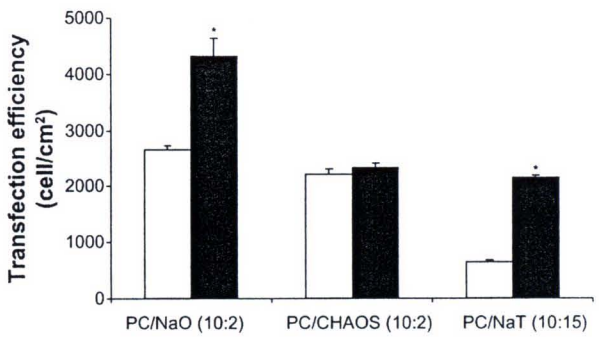

Figure 6 Comparison of the transfection efficiencies of PCL/DNA complexes formulated with PC/NaO (10:2), PC/CHAPS (10:2), and PC/NaT (10:1.5) in Huh7 cells with 10% serum (□) and without serum (■) in the transfection reagent.

Note: * statistically significant ($P < 0.05$).

Abbreviations: PC, phosphatidylcholine; PCL, polycationic liposomes; NaO, sodium oleate; NaT, sodium taurocholate; CHAPS (3-[{3-cholamidopropyl}-dimethylammonio]-1-propanesulfonate) (zwitterionic surfactant).

Conclusion

In this study, we have developed PEI-coated anionic liposomes as efficient gene carriers. PCL composed of PC:NaO (10:2) showed superior transfection efficiency with NaT and CHAPS at all weight ratios tested, while for PC/CHAPS the transfection capability did not alter even in the presence of serum. This study suggests that PCL is safe and exhibits a significantly improved gene delivery capability in vitro. Because both PEI and liposomes can be easily modified with various ligands, these lipopolyplexes might be useful in the design of targeted carriers specific for cell-surface receptors in the delivery of therapeutic genes.

Acknowledgments

The authors would like to acknowledge the financial support of the Research, Development and Engineering Fund through the National Nanotechnology Center, National Science and Technology Development Agency, Thailand (Project No. NN-B-22-EN3-17-52-10), and Commission of Higher Education (Thailand), The Thailand Research Fund (DBG5180005) through the Golden Jubilee Ph.D. Program (Grant No. PHD/0092/2551), and Silpakorn University Research and Development Institute (SURDI 53/01/37).

Disclosure

The authors report no conflicts of interest in this work.

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