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APPENDICES

Appendix A Electrical conductivity of proteins-loaded Hydroxyapatite powder

Twenty milliliter of the suspension was placed in an electrophoresis cup, and the movable images of the particles were recorded by the electrophoresis apparatus. The zeta potentials of all the samples are negative, indicating that the particles themselves have superfluous positive electrical charges.

Table A1 Raw data of electrical conductivity of proteins-loaded Hydroxyapatite

Electrical Conductivity					
	Neat HAp 1	Neat HAp 2	HAp+OVA 1	HAp+OVA 2	HAp+GelB 1
1	-20.0	-18.1	-20.4	-13.3	-14.3
2	-22.6	-19.8	-19.2	-17.8	-18.9
3	-16.7	-19.0	-18.1	-11.8	-17.6
4	-13.7	-18.1	-12.6	-17.0	-23.8
5	-12.8	-19.8	-13.6	-14.3	-21.1
6	-12.7	-19.0	-23.2	-14.9	-23.8
7	-14.7	-18.1	-17.7	-18.2	-21.1
8	-14.6	-18.6	-20.4	-12.1	-23.8
9	-13.3	-22.3	-15.0	-14.1	-14.3
10	-14.9	-14.0	-12.3	-19.2	-22.6
Average	-17		-16		-20

Appendix B Proteins release from HAp powder and HAp-PCL scaffold

1. Proteins release from HAp powder

The proteins release from hydroxyapatite was assessed in two different environments under phosphate buffer silane solution pH 7.4 and medium for cell culture condition. In this study, the 4 types of proteins-loaded hydroxyapatite : Ovalbumin, Gelatin type B, Bovine serum albumin and Crude bone protein extraction were examined to observe a change in release rate.

% Protein Release was measured by UV-Visible spectroscopy at 280 nm for OVA and BSA, 275 nm for CBP and 215 nm for Gelatin type B and calculated by the following equation.

$$\text{Encapsulating efficiency (\%)} = \frac{\text{total mg proteins-encapsulated}}{\text{initial mg proteins-loaded}} \times 100$$

$$\text{Loading capacity (\%)} = \frac{\text{total mg proteins-encapsulated}}{\text{total mg particles}} \times 100$$

2. Proteins release from HAp-PCL scaffold

The proteins/HAp-PCL were immersed in 1 ml of the minimum essential medium (MEM; Gibco, Invitrogen) in 24 well plate. All samples were incubated in a shaking water bath (70 rpm) at 37°C. The amount of proteins releasing from scaffold to the supernatant was measured by BCA protein assay at various times. After the releasing medium (sample solution) was withdrawn 20 µl to mixed with BCA solution, an equal amount of fresh medium was added to maintain a constant volume of the medium. The amount of protein in the sample solution was determined by UV-Visible spectroscopy at 562 nm.

$$\text{Encapsulating efficiency (\%)} = \frac{\text{total mg proteins-encapsulated}}{\text{initial mg proteins-loaded}} \times 100$$

Table B1 Raw data of the proteins release from HAp powder

% Protein Release from HAp												
Day	HAp/ OVA 1	HAp/ OVA 2	Average	HAp/ GelB 1	HAp/ GelB 2	HAp/ GelB 3	Average	HAp/ BSA 1	HAp/ BSA 2	HAp/ BSA 3	Average	HAp/ CBP 1
0	0	0	0	0	0	0	0	0	0	0	0	0
1	4.296	4.191	4.244	2.501	1.898	2.293	2.230	0.562	0.334	0.889	0.595	4.319
4	12.381	12.575	12.478	5.644	5.520	5.631	5.599	1.539	1.243	6.606	3.129	13.226
7	17.692	19.314	18.503	8.897	9.275	9.095	9.089	6.354	2.974	13.516	7.615	23.321
14	30.355	29.495	29.925	11.620	12.556	11.965	12.047	14.187	8.355	21.108	14.550	n/a
21	41.802	39.318	40.560	11.620	12.556	11.965	12.047	22.431	18.183	30.889	23.834	41.211
28	51.930	48.681	50.306	11.620	12.556	11.965	12.047	33.235	32.392	42.280	35.969	52.648
35	51.930	48.681	50.306	11.620	12.556	11.965	12.047	42.528	47.373	53.671	47.857	52.648
42	51.930	48.681	50.306	11.620	12.556	11.965	12.047	50.495	60.528	64.004	58.342	52.648

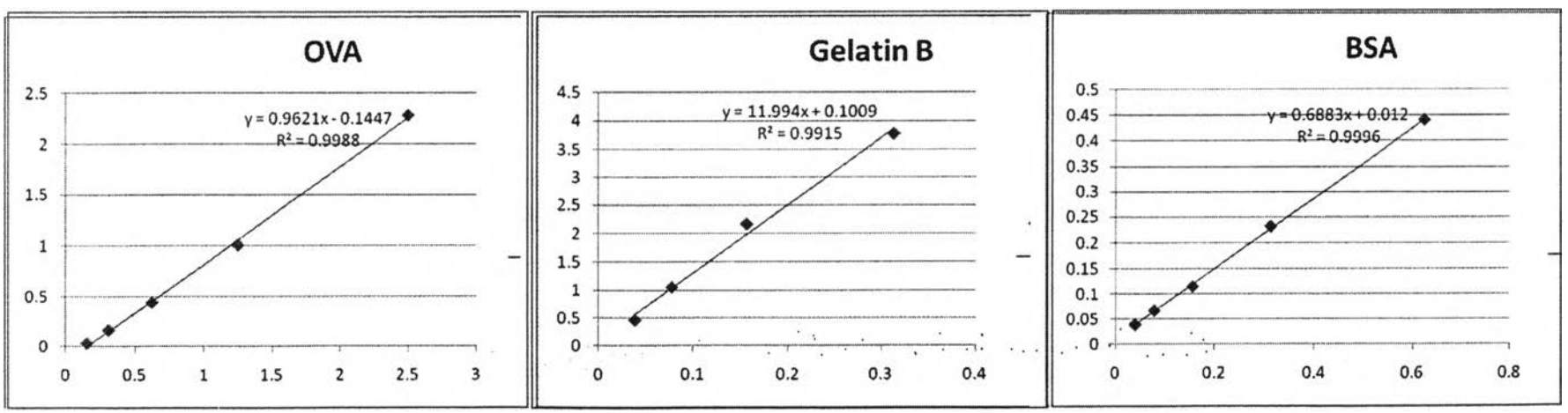
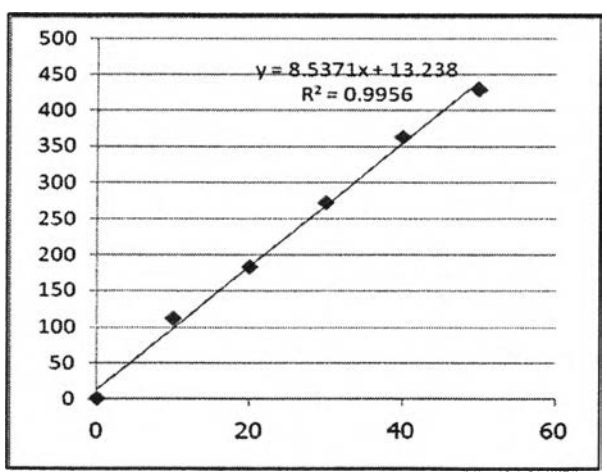


Table B2 Raw data of the proteins release from HAp-PCL scaffolds

Day	HAp/ OVA 1	HAp/ OVA 2	Average	HAp/ GelB 1	HAp/ GelB 2	Average	HAp/ BSA1	HAp/ BSA2	Average	HAp/ CBP1	HAp/ CBP2	Average
4	0.262	0.296	0.279	0.366	0.362	0.364	0.294	0.263	0.279	n/a	n/a	n/a
7	0.619	0.527	0.573	0.782	0.739	0.761	0.607	0.59	0.599	1.49	1.50	1.50
14	0.891	0.906	0.899	0.982	1.002	0.992	0.915	0.964	0.940	2.129	2.203	2.166
21	1.784	1.654	1.719	2.044	1.995	2.0195	1.869	1.877	1.873	2.144	2.139	2.142

BCA standard curve



Appendix C Particle size distribution of protein-loaded hydroxyapatite powder

Table C1 Raw data of particle size distribution of HAp/OVA

Size (μm)	Volume In %	Size (μm)	Volume In %	Size (μm)	Volume In %	Size (μm)	Volume In %	Size (μm)	Volume In %
0.01	0	0.182	0	3.311	0.07	60.256	0.82	1096.478	0.06
0.011	0	0.209	0	3.802	0.07	69.183	1.14	1258.925	0
0.013	0	0.24	0	4.365	0.07	79.433	1.53	1445.44	0
0.015	0	0.275	0	5.012	0.07	91.201	1.97	1659.587	0
0.017	0	0.316	0	5.754	0.07	104.713	2.47	1905.461	0
0.02	0	0.363	0	6.607	0.07	120.226	3.02	2187.762	0
0.023	0	0.417	0	7.56	0.07	138.038	3.62	2511.886	0
0.026	0	0.479	0	8.71	0.07	158.489	4.31	2884.032	0
0.03	0	0.55	0	10	0.07	181.97	5.07	3311.311	0
0.035	0	0.631	0	11.482	0.08	208.93	5.9	3801.894	0
0.04	0	0.724	0	13.183	0.09	239.883	6.71	4365.158	0
0.046	0	0.832	0	15.136	0.09	275.423	7.46	5011.872	0
0.052	0	0.955	0	17.378	0.1	316.228	8.03	5754.399	0
0.06	0	1.096	0	19.953	0.1	363.078	8.32	6606.934	0
0.069	0	1.259	0	22.909	0.11	416.869	8.23	7585.776	0
0.079	0	1.445	0	26.303	0.11	478.63	7.73	8709.636	0
0.091	0	1.66	0	30.2	0.13	549.541	6.83	10000	0
0.105	0	1.905	0	34.674	0.17	630.57	5.62		
0.12	0	2.188	0	39.811	0.25	724.436	4.21		
0.138	0	2.512	0	45.709	0.37	831.764	2.85		
0.158	0	0.884	0.05	52.481	0.56	954.993	1.28		

Appendix D Characterization of Polyscprolactone scaffold

The density of the scaffolds ($\rho_{scaffold}$) is determined by using a Sartorius YDK01, Germany density measurement kit (Buoyancy method) which can be calculated using the following equation.

$$\rho_{scaffold} = \frac{W_a \times \rho_{fl}}{W_a \times W_{fl}}$$

Table D1 Raw Data of the density of the PCL scaffold.

	1	2	3	4	5	6	7	8	9
W(a)	0.046	0.047	0.047	0.047	0.045	0.044	0.048	0.047	0.046
W(a)-W(fl)	0.393	0.426	0.421	0.413	0.384	0.416	0.422	0.377	0.428
ρ(fl)	0.998	0.998	0.998	0.998	0.997	0.997	0.997	0.997	0.997
ρPCLscaffold	0.117	0.110	0.111	0.114	0.117	0.107	0.115	0.124	0.109
Average	0.1139 g/cm^3								

Appendix E Experimental data of water absorption

The dry scaffold scaffolds were weighted and then were immersed in 5 ml of phosphate buffer silane solution (PBS pH 7.4) solution at room temperature for 3 days. Scaffolds were removed from the solution and carefully placed on the glass for 5 seconds to remove the excessive water and weight immediately. The water absorption was calculated using the following equation.

$$\text{Water absorption (\%)} = \frac{(M_{wet} - M_{dry})}{M_{dry}} \times 100$$

Where M_{dry} and M_{wet} are the weight of the scaffold before and after immersion in water respectively. Five measurements were performed for the calculation of an average water absorption value.

Table E1 Raw data of water absorption of PCL scaffold

	1	%water absorption	2	%water absorption	3	%water absorption	Average
0	0.044	0	0.0454	0	0.0456	0	0
1	0.2021	78.2286	0.2097	78.35002	0.2162	78.90842	78.49568
2	0.2322	81.05082	0.2300	80.26087	0.235	80.59574	80.63581
3	0.2486	82.30088	0.2436	81.36289	0.2545	82.08251	81.91543
4	0.2563	82.83262	0.255	82.19608	0.2639	82.72073	82.58314
5	0.2759	84.05219	0.2717	83.29039	0.2715	83.20442	83.51567
6	0.2874	84.69033	0.2833	83.97459	0.2826	83.86412	84.17634
7	0.2996	85.31375	0.2994	84.83634	0.2861	84.06152	84.7372
8	0.306	85.62092	0.3019	84.96191	0.2978	84.68771	85.09018
9	0.3141	85.99172	0.3178	85.71429	0.3084	85.21401	85.64001
15	0.345	87.24638	0.3465	86.89755	0.3274	86.07208	86.73867
24	0.3589	87.74032	0.3789	88.01795	0.3565	87.20898	87.65575
48	0.3884	88.67147	0.391	88.38875	0.401	88.62843	88.56288
72	0.4327	89.83129	0.414	89.03382	0.4574	90.03061	89.63191

Appendix F Experimental data of cell culture studies

The potential for use of HAp-PCL as bone scaffolds was assessed by mouse calvaria derived pre-osteoblastic cells, MC3T3-E1, in terms of indirect cytotoxicity, cell attachment, cell proliferation, alkaline phosphatate (ALP) activity, and mineralization.

Table F1 Raw data of direct cytotoxicity test of proteins determinate the viability of MC3T3-E1 by MTT assay at 570 nm

	Control			OVA			Gelatin type B			BSA			CBP		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	0.364	0.504	0.663	0.374	0.509	0.593	0.365	0.493	0.628	0.373	0.475	0.665	0.319	0.488	0.564
2	0.363	0.495	0.664	0.38	0.504	0.561	0.362	0.519	0.61	0.359	0.493	0.62	0.3	0.457	0.539
3	0.364	0.481	0.664	0.397	0.492	0.578	0.387	0.507	0.594	0.367	0.534	0.589	0.292	0.453	0.519
Average	0.364	0.493	0.664	0.384	0.502	0.577	0.371	0.506	0.611	0.366	0.501	0.625	0.304	0.466	0.541

Table F2 Raw data of indirect cytotoxicity test of protein-loaded hydroxyapatite powder determinate the viability of MC3T3-E1 by MTT assay at 570 nm cytotoxicity of hydroxyapatite powder

	Control			Neat HAp			HAp+OVA			HAp+Gelatin type B			HAp+BSA		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	0.334	0.337	0.434	0.341	0.335	0.413	0.341	0.371	0.416	0.349	0.369	0.426	0.388	0.368	0.443
2	0.317	0.322	0.413	0.344	0.352	0.416	0.344	0.378	0.418	0.358	0.373	0.408	0.371	0.373	0.427
3	0.344	0.339	0.416	0.359	0.345	0.412	0.359	0.376	0.429	0.364	0.37	0.431	0.393	0.373	0.448
Average	0.332	0.333	0.421	0.348	0.344	0.414	0.348	0.375	0.421	0.357	0.371	0.422	0.384	0.371	0.439

Table F3 Raw data of indirect cytotoxicity test of protein-loaded hydroxyapatite –PCl scaffold determinate the viability of MC3T3-E1 by MTT assay at 570 nm Cytotoxicity of Hydroxyapatite powder Cytotoxicity of HAp-PCL scaffold

	Control		PCL		PCL+Neat HAp		PCL+OVA		PCL+Gelatin type B		PCL+BSA	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	0.339	0.548	0.361	0.504	0.334	0.571	0.347	0.55	0.379	0.623	0.376	0.57
2	0.345	0.552	0.317	0.519	0.319	0.527	0.32	0.581	0.334	0.63	0.337	0.578
3	0.341	0.555	0.34	0.541	0.329	0.523	0.335	0.6	0.36	0.646	0.36	0.593
Average	0.342	0.552	0.339	0.521	0.327	0.540	0.334	0.577	0.358	0.633	0.358	0.580

Table F4 Raw data of cell attachment of HAp-PCL scaffold, determined the viability of MC3T3-E1 by MTT assay at 570 nm

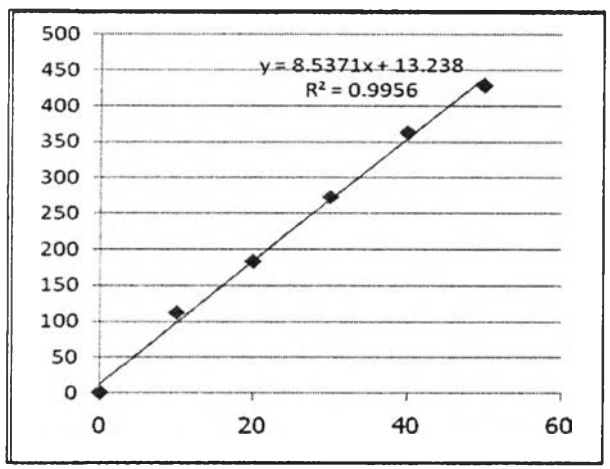
	Control			PCL			HAp-PCL			OVA/HAp-PCL			Gelatin type B/HAp-PCL			BSA/HAp-PCL			CBP/HAp-PCL		
	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h
1	0.199	0.392	0.397	0.131	0.209	0.332	0.163	0.261	0.317	0.161	0.215	0.345	0.179	0.229	0.295	0.137	0.172	0.379	0.162	0.278	0.347
2	0.210	0.380	0.409	0.142	0.223	0.319	0.160	0.248	0.336	0.149	0.221	0.340	0.173	0.236	0.299	0.133	0.161	0.386	0.147	0.269	0.314
3	0.205	0.376	0.429	0.127	0.218	0.310	0.155	0.237	0.313	0.140	0.207	0.314	0.190	0.248	0.310	0.134	0.170	0.354	0.151	0.283	0.319
Average	0.204	0.382	0.412	0.133	0.217	0.32	0.159	0.249	0.322	0.15	0.214	0.333	0.18	0.237	0.301	0.135	0.168	0.373	0.153	0.277	0.327

Table F5 Raw data of cell proliferation of HAp-PCL scaffold, determined the viability of MC3T3-E1 by MTT assay at 570 nm

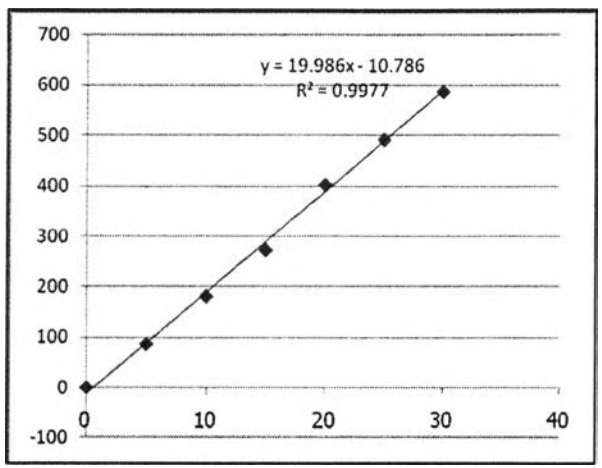
	Control		PCL		HAp-PCL		OVA/HAp-PCL		Gelatin type B/HAp-PCL		BSA/HAp-PCL		CBP/HAp-PCL	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
1	0.511	0.526	0.306	0.325	0.325	0.331	0.323	0.302	0.378	0.328	0.339	0.344	0.348	0.325
2	0.523	0.545	0.341	0.310	0.336	0.312	0.340	0.320	0.362	0.316	0.327	0.320	0.323	0.313
3	0.510	0.527	0.325	0.300	0.341	0.301	0.335	0.314	0.356	0.315	0.347	0.308	0.329	0.303
Average	0.515	0.532	0.324	0.311	0.334	0.314	0.333	0.312	0.366	0.32	0.338	0.324	0.334	0.314

Table F6 Raw data of ALP activity of MC3T3-E1 seeded on proteins, PCL, HAp-PCL, and protein-loaded HAp-PCL scaffold. Amount of protein was determinate with BCA protein assay.

BCA standard curve



ALP standard curve



BCA 3 d.	Absorbance	µmol/µl	ALP 3 d.	Absorbance	µmol/µl	ALP activity
Control	0.167	18.01103	Control	0.167	8.895527	0.493893
Control	0.202	22.11079	Control	0.151	8.094966	0.366109
PCL	0.455	51.74614	PCL	0.478	24.45642	0.472623
PCL	0.325	36.51849	PCL	0.296	15.35005	0.420336
HAp-PCL	0.431	48.93488	HAp-PCL	0.262	13.64885	0.278919
HAp-PCL	0.291	32.53587	HAp-PCL	0.191	10.09637	0.310315
OVA/HAp-PCL	0.566	64.74822	OVA/HAp-PCL	0.364	18.75243	0.289621
OVA/HAp-PCL	0.49	55.8459	OVA/HAp-PCL	0.284	14.74962	0.264113
Gel B/HAp-PCL	0.511	58.30575	Gel B/HAp-PCL	0.178	9.445912	0.162007
Gel B/HAp-PCL	0.445	50.57479	Gel B/HAp-PCL	0.186	9.846192	0.194686
BSA/HAp-PCL	0.435	49.40343	BSA/HAp-PCL	0.308	15.95047	0.322862
BSA/HAp-PCL	0.501	57.13439	BSA/HAp-PCL	0.383	19.70309	0.344855
CBP/HAp-PCL	0.535	61.11701	CBP/HAp-PCL	0.399	20.50365	0.335482
CBP/HAp-PCL	0.478	54.44027	CBP/HAp-PCL	0.364	18.75243	0.344459

BCA 7 d.	Absorbance	µmol/µl	ALP 7 d.	Absorbance	µmol/µl	ALP activity
Control	0.261	29.0218	Control	0.173	9.195737	0.316856
Control	0.747	85.9498	Control	0.654	33.26258	0.387
PCL	0.651	74.70476	PCL	0.411	21.10407	0.2825
PCL	0.793	91.33804	PCL	0.402	20.65376	0.226124
HAp-PCL	0.691	79.39019	HAp-PCL	0.352	18.15201	0.228643
HAp-PCL	0.379	42.84382	HAp-PCL	0.161	8.595317	0.20062
OVA/HAp-PCL	0.751	86.41834	OVA/HAp-PCL	0.463	23.70589	0.274316
OVA/HAp-PCL	0.539	61.58555	OVA/HAp-PCL	0.323	16.70099	0.271184
Gel B/HAp-PCL	0.524	59.82851	Gel B/HAp-PCL	0.288	14.94976	0.249877
BSA/HAp-PCL	0.687	78.92165	BSA/HAp-PCL	0.642	32.66216	0.413856
BSA/HAp-PCL	0.651	74.70476	BSA/HAp-PCL	0.481	24.60652	0.329384
CBP/HAp-PCL	0.675	77.51602	CBP/HAp-PCL	0.481	24.60652	0.317438

Table E7 Raw data of ALP activity of MC3T3-E1 determined on different proteins

BCA 5 d	Absorbance	µmol/µl	ALP 5 d	Absorbance	µmol/µl	ALP activity
Control	0.163	17.54249	Control	0.193	10.19644	0.581242
Control	0.16	17.19108	Control	0.149	7.994896	0.465061
OVA	0.148	15.78545	OVA	0.129	6.994196	0.443079
OVA	0.138	14.6141	OVA	0.125	6.794056	0.464897
Gelatin type B	0.171	18.47958	Gelatin type B	0.15	8.044931	0.435342
Gelatin type B	0.173	18.71385	Gelatin type B	0.119	6.493846	0.347007
BSA	0.158	16.95681	BSA	0.175	9.295807	0.548205
BSA	0.164	17.65963	BSA	0.15	8.044931	0.455555
CBP	0.159	17.07395	CBP	0.093	5.192935	0.304144
CBP	0.092	9.22585	CBP	0.061	3.591814	0.389321

BCA 7 d	Absorbance	µmol/µl	ALP 7 d	Absorbance	µmol/µl	ALP activity
Control	0.173	18.71385	Control	0.134	7.244371	0.387113
Control	0.162	17.42536	Control	0.124	6.744021	0.387023
OVA	0.174	18.83098	OVA	0.111	6.093565	0.323593
OVA	0.169	18.24531	OVA	0.105	5.793355	0.317526
Gelatin type B	0.129	13.55987	Gelatin type B	0.073	4.192235	0.309165
Gelatin type B	0.13	13.67701	Gelatin type B	0.088	4.94276	0.361392
BSA	0.15	16.01973	BSA	0.093	5.192935	0.324159
BSA	0.196	21.40797	BSA	0.126	6.844091	0.319698
CBP	0.095	9.577257	CBP	0.095	6.243671	0.376002
CBP	0.108	11.10002	CBP	0.066	3.841989	0.346124

Table E8 Raw data of mineralization of MC3T3-E1 determined on TCPS, PCL, HAp-PCL, and protein-loaded HAp scaffold by using 10% cetylpyridinium chloride for eluted dye of alizarin Red-S

21 d		21 d	
PCL no cell	0.363	OVA/HAp-PCL	1.418
HAp-PCL no cell	1.584	GelatinB/HAp-PCL	2.268
TCPS	0.043	GelatinB/HAp-PCL	1.763
TCPS	0.047	BSA/HAp-PCL	1.502
Neat PCL	0.828	BSA/HAp-PCL	1.423
Neat PCL	0.799	CBP/HAp-PCL	3.238
HAp-PCL	2.695	CBP/HAp-PCL	3.196
HAp-PCL	2.722		
OVA/HAp-PCL	1.544		

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Presentations:

1. Chaisuntharanon, S.; Pavasant, P.; Kuanchertchoo, N.; and Supaphol, P. (2010, April 22) Development of Porous Hydroxyapatite Particles as Carriers of Proteins in a Polycaprolactone Scaffold for Bone Tissue Engineering. Paper Presented at the 16th PPC Symposium on Petroleum, Petrochems, and Polymers, Bangkok, Thailand.