Bone Targeting Prodrugs Based on Peptide Dendrimers, Synthesis and Hydroxyapatite Binding *In Vitro*

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Abstract: Novel bone targeting naproxen prodrugs with poly(aspartic acid) moieties and with two and three poly(aspartic acid) sequences peptide dendrimers were synthesized using a conventional method. The modified naproxen conjugates were incubated with hydroxyapatite in PBS at physiological conditions over 16h. The study revealed the hydroxyapatite binding properties of poly(aspartic acid) and it was found that the peptide dendrimer prodrugs exhibited a faster initial binding and a greater total binding. The obtained binding data *in vitro* indicated that the peptide dendrimers with poly(aspartic acid) sequences were useful for the development of new bone targeting molecules for drug delivery to bone.

Keywords: Bone-targeting, poly(aspartic acid), peptide dendrimers, hydroxyapatite, *in vitro*.

INTRODUCTION

Nowadays, musculoskeletal diseases such as ostearthritis and osteoporosis are recognized as major public health problems in the world. There is a great interest in the development of drug delivery systems that could allow an efficient transport of drugs to the bone. Bone differs from the rest of the body by the presence of hydroxyapatite(HAP). We have known that tetracyclines and bisphosphonates exhibit a strong affinity to HAP and can be useful as targeting moieties to bone [1,2].

In recent years, acidic oligopeptides such as poly-aspartic acid (Asp_n) have been reported as novel bone targeting drug carriers [3,4]. Unlike the P-C-P bond of bisphosphonates, the Asp_n are biologically labile and enzymatically degraded. The advantageous property can make more efficient drug release and there is no unexpected long-term effects of the bone targeting moiety [5]. Therefore, the Asp_n seems very attractive as an alternative bone targeting carrier to bisphosphonates. Previous work has been conducted that estradiol modified with Asp_6 exhibited high accumulation in bone *in vivo* [6], while polymers PEG and HPMA conjugates to Asp_8 showed greater than 80% binding to HAP *in vitro* [7]. However, there is few literature for the relationship between the numbers of the negatively charged amino acid residues in Asp_n and their respectively HAP binding rates.

Anti-inflammatory drugs (NSAIDs) are clinically used in ostearthritis. However, they have been reported to cause a number of side effects, in particular gastrointestial toxicity [8]. In this present study, to improve the therapeatic index of NSAIDs, we selected $Asp_{(4-6)}$ as bone targeting moieties and developed a type of conjugates using a NSAID naproxen(NAP) as prodrugs through an amide linkage. We hoped that they could show selective targeting to HAP and we could investigate the different HAP binding rates of the different length of the oligopeptides' chain (Asp_4, Asp_5, Asp_6). The structures of conjugates were shown in Fig. (**1**).

Furthermore, on the basis of peptide dendrimers: peptide dendrimers are synthetic, highly branched, spherical, monodisperse macromolecules. They have a protein-like globular structure, good biocompatibility and biodegradability which make them macromolecular vehicles for drug delivery purposes and other biomedical application [9]. A dendritic linker approach will increase the molar ratio of Asp_n, which may increase the bone affinity and more negative charges in dendrimer periphery increase the binding capacity to the HAP crystal surface [10,11]. The increased molar ratio can be controlled by varying the size or generation of the dendritic molecule, which will allow investigations into relationship between the molar ratio of targeting moieties and bone targeting abilities of conjugates.

In this manuscript, we reported the design and synthesis of the first generation dendritic compounds with two or three $Asp_{(4-6)}$ fragments. We employed the polyamide and polyether-amide dendrimers as scaffolds which were successfully used in the RGD and CCK peptide dendrimers in our previous work [12]. These dendrimers have an flexible scaffold, biocompatibility and low cytotoxicity and these characters were helpful in the drug delivery. The structure of these peptide dendrimers were shown in Fig. (**1**). We aimed to increase the bone-targeting potency of the peptide dendrimers with a dendritic approach. The HAP binding assay was established *in vitro* condition and the affinity of the targeting conjugates for HAP was reported here.

EXPERIMENTAL SECTION

General Remarks

All starting materials, unless otherwise specified, were used as received. The compounds **13**,**14** were synthesized

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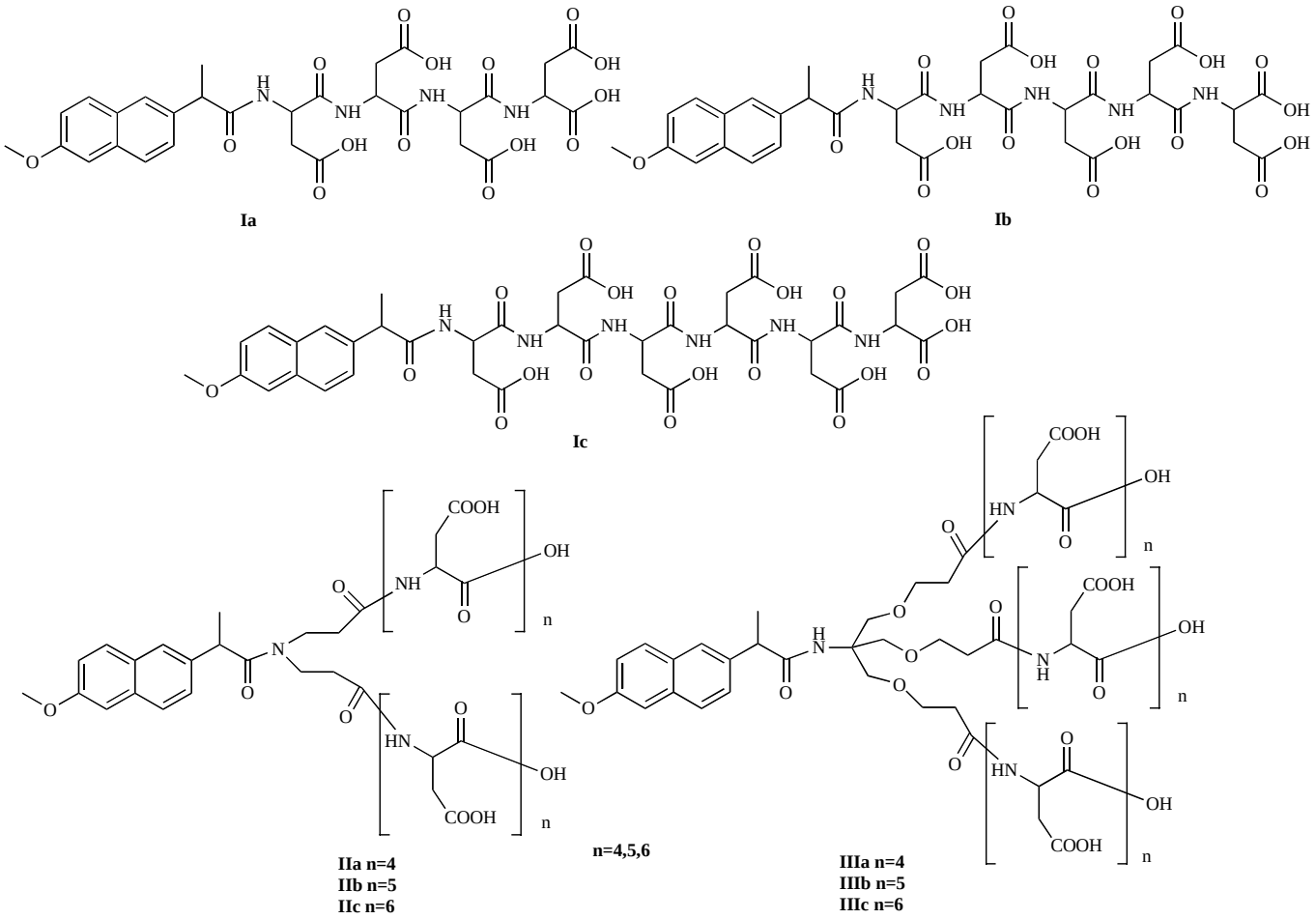


Fig. (1). The structures of the bone targeting prodrugs.

according to the literature method, respectively [13]. TLC was performed on glass-backed silica plates. ^{1}H NMR analysis was performed by the INOVA VARIAN 400 MHz spectrometer using $CDCl_3$ or D_2O as a solvent at room temperature. Mass spectra were recored on a Agilent 1946B ESI-MS instrument. The synthetic procedure was outline in Scheme **1** and Scheme **2**.

Synthetic Procedure for Asp$_{(4-6)}$ Peptide 6a-c

The bone-targeting peptides were synthesized by a conventional liquid phase peptides synthetic method from Boc-Asp-Obzl utilizing IBCF(isobutyl chloroformate) and NMM(N-methyl morpholine)(mixed anhydrides method) by a series of segment condensation. The -COOH part dissolved in anhydrous THF was cooled at -15˚C, then added 1eq. NMM and IBCF, after stirred for 5 min, the -NH_2 part in THF was added to the reaction mixture. After the coupling reaction was completed at room temperature. The mixture was purified by recrystallisation or silica column chromatography using $CHCl_2$ and CH_3OH as an eluent, then deprotected in trifluoroacetic acid to give the peptides, total yield: 6a, five steps reactions 47%; 6b, seven steps reactions, 34%; 6c, nine steps reactions 23%.

Data: 6a: ESI-MS(*m/z*): calcd. for 929.4 ($[M+H]^+$), obsd. 929.3

6b: ESI-MS(*m/z*): calcd. for 1133.4 ($[M+H]^+$), obsd. 1134.2

6c: ESI-MS(*m/z*): calcd. for 1339.5 ($[M+H]^+$), obsd. 1339.7

Synthetic Procedure for Compound 8

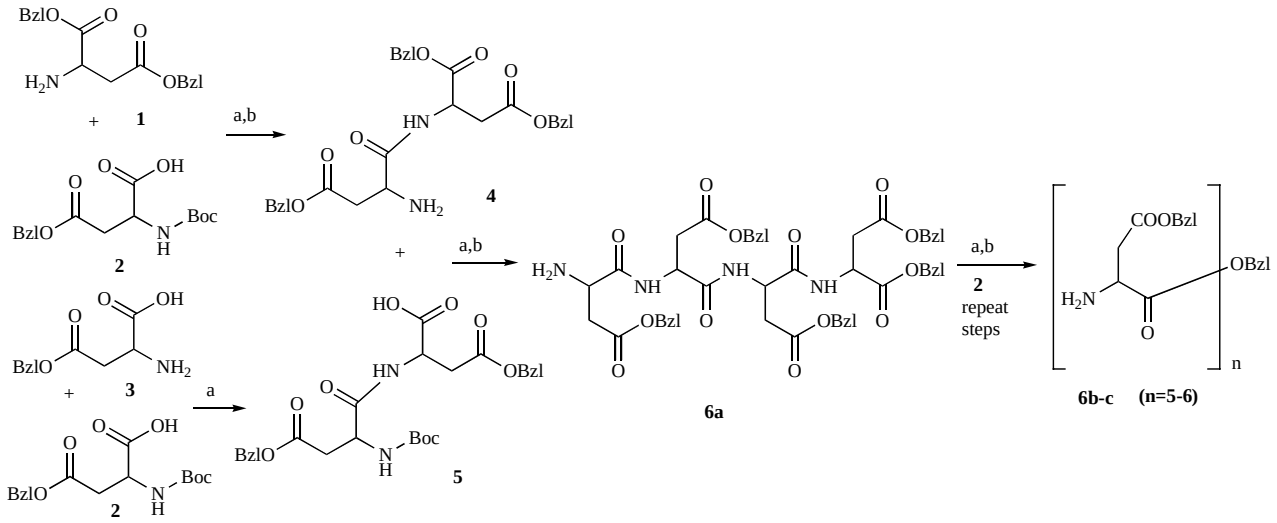
A mixture of ethyl acrylate (10g,100mmol), benzylamine (4g, 37mmol) in CH_3OH (10ml) was stirred in room temperature for 48 hours. The solvent was evaporated and the residue was diluted with ethyl acetate (50ml). After washed with saturated NaCl(15ml×3), the organic layer was dried over anhydrous Na_2SO_4 and evaporated to yield a light-yellow oil (8) 9.6g, yield 84%.

Synthetic Procedure for Compound 9

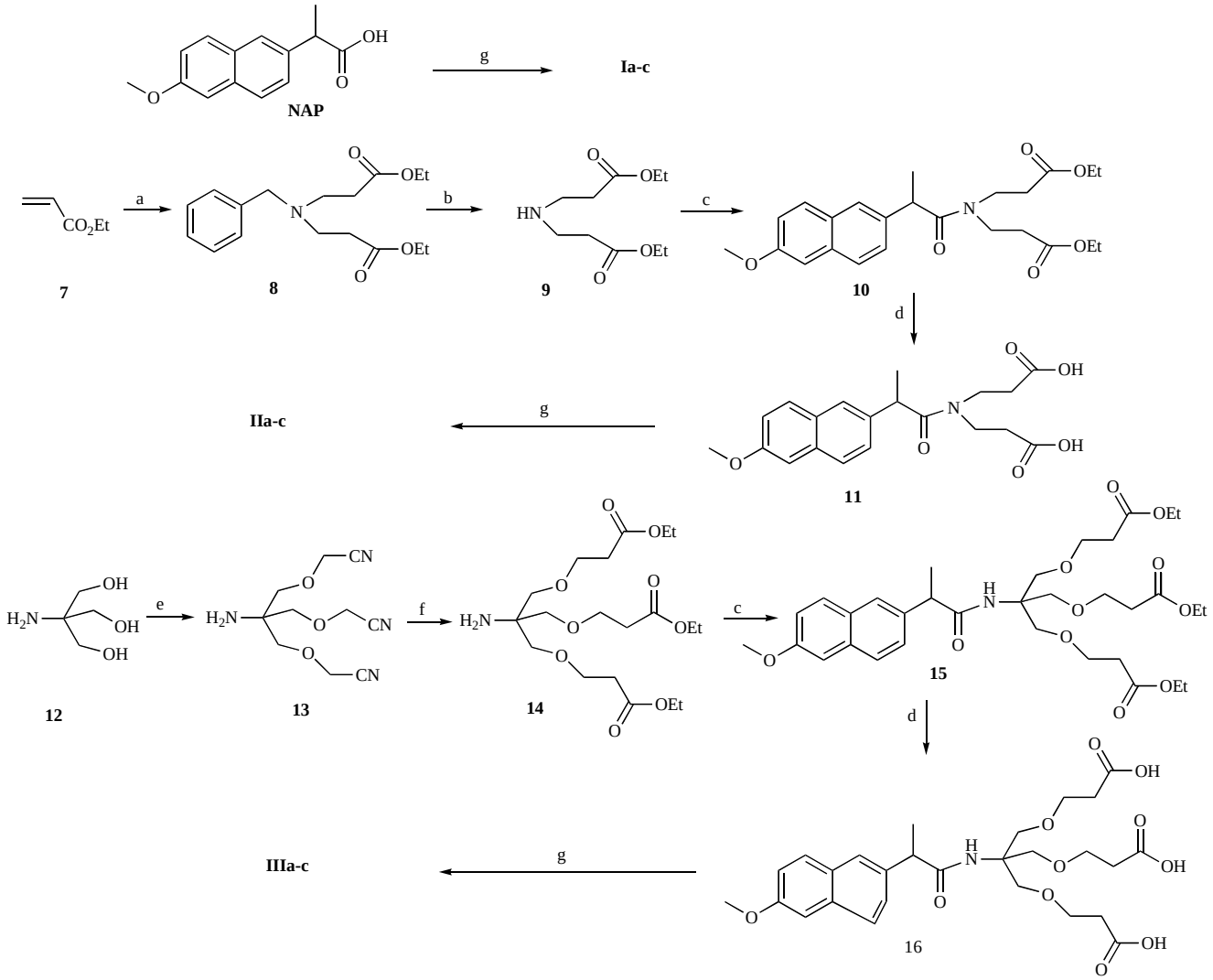
Compound **8** (5g,16mmol) was dissolved in anhydrous CH_3OH (20ml), ammonium formate(5g,79mmol) and 10% Pd/C(450mg) was added.The mixture was refluxed for 2h. Then it was allowed to attain room temperature and filter. Following removal of the solvent, a colorless oil(9) was obtained 3.0g, yield 85%.

Synthetic Procedure for Compound 10

A stirred solution of naproxen (2.3g, 10mmol) in THF (10ml) was added DCC (2.06,10mmol) and HoSu (1.15g,10mmol) at 0˚C, after stirred for 3h, 9(2.17g, 10mmol) was added at room temperature for 24h.The dicy-



Scheme 1. Synthetic procedure of the $Asp_{(4-6)}$ peptides.
Reagents and Conditions: **a**) IBCF, NMM, THF; **b**)TFA, CH_2Cl_2.



Scheme 2. Synthetic procedure of the Nap-Peptide dendrimers

Reagents and Conditions: **a**) $BnNH_2$, CH_3OH, r.t; **b**) ammonium formate, CH_3OH, reflux; **c**) Naproxen, DCC, HoSu, THF, r.t; **d**) 2N NaOH/CH_3OH, r.t; **e**) acrylonitrile, 40%KOH, 0˚C; **f**) H_2SO_4, C_2H_5OH, reflux; **g**) i, **6a-c**, DCC, HoBt, THF, r.t; ii, 10% Pd/ C, CH_3OH, r.t.

clohexylurea (DCU) was removed by filtration. The filtrate was purified by silica columnn chromatography using EA and PE as an eluent to afford a colorless oil (10), 2.8g, yield 66%.

Data: ^{1}HNMR (400MHz, $CDCl_3$): δ=1.28 (t, 6H, J=6.4Hz $OCH_2\mathbf{CH_3}$×2), 1.52(d, 3H, J=7.2Hz, NAP**CH_3**), 2.38 (t, 4H, J=6, **CH_2**CO×2), 3.52(t, 4H, J=6Hz, N-**CH_2**×2), 3.89(brs, 4H, NAPO**CH_3+CH**), 4.17 (m, 4H, O**CH_2**CH_3×2), 7.10-7.7 (m, 6H, NAP**ph-H**).

Synthetic Procedure for Compound 11

Compound **10** (1.0g, 2mmol) was dissolved in CH_3OH(10ml), aqueous NaOH(2mol/L) was added. The mixture was stirred for 5h, then acidified by HCl(2mol/L) to pH<3. The solvent was evaporated and the residue was diluted with ethyl acetate. (15ml×3). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to yield a white powder(11), 0.77g, yield: 90%.

Data: ^{1}HNMR (400MHz, $CDCl_3$): δ=1.53(d, 3H, J=7.2Hz, NAP**CH_3**), 2.41(t, 4H, J=6Hz **CH_2**CO×2), 3.47 (t, 4H, J=6Hz N-**CH_2**×2), 3.89(brs, 4H, NAP**CH_3**O+**CH**), 7.10-7.7(m, 6H NAP**ph-H**).

ESI-MS(*m/z*): calcd. for 374.4 ($[M+H]^+$), obsd. 374.1

Synthetic Procedure for Compound 15

Same procedure as describe above for preparation of the compound 10. A colorless oil, yield: 71%.

Data: ^{1}HNMR(400MHz, $CDCl_3$): δ=1.31(t, 9H, J=6.4, $OCH_2\mathbf{CH_3}$×3), 1.52(d, 3H, J=7.2Hz, NAP**CH_3**), 2.38(brs, 6H, **CH_2**CO×3), 3.52-3.7(m, 6H, O**CH_2**×3), 3.91(brs, 4H, NAP**CH_3**O+**CH**), 4.14(m, 6H, O**CH_2**CH_3×3), 7.10-7.7(m, 6H NAP**ph-H**)

Synthetic Procedure for Compound 16

Same procedure as describe above for preparation of the compound 11. A colorless oil. yield 95%.

Data: ^{1}HNMR(400MHz, $CDCl_3$): 1.52(d, 3H, J=7.2Hz, NAP**CH_3**), 2.41 (brs, 6H, **CH_2**CO×3), 3.42-3.7(m, 6H, O**CH_2**×3), 3.91(brs, 4H, NAP**CH_3**O+**CH**), 7.10-7.7(m, 6H, NAP**ph-H**).

ESI-MS(*m/z*): calcd. for 550.6 ($[M+H]^+$), obsd. 550.4

Synthetic Procedure for Ia-c

The $Asp_{(4-6)}$ peptides 6a-c(6a, 0.9g, 1.0 mmol; 6b, 1.1g, 1.0 mmol; 6c, 1.3g, 1.0mmol) dissolved in anhydrous THF(10ml). Naproxen (0.23g, 1.0mmol) and HOBt (0.15g, 1.1mmol) were added to the reaction mixture. DCC (0.23g, 1.1mmol) dissolved in THF was slowly dropped into the solution. After the coupling reaction was completed at room temperature, the solution was filtered twice to remove the DCU. The filtrate was purified by silica column chromatography using $CHCl_2$ and CH_3OH as an eluent, then deprotected the -Obzl with Pd catalyst by hydrogen in CH_3OH at room temperature to afford **Ia-c** (**Ia**, 0.44g, yield 63%; **Ib**, 0.45g, yield 56%;**Ic**, 0.53g, yield 58%) as white powders.

Data: **Ia**: ^{1}HNMR (400MHz, D_2O): δ=1.52(d, 3H, J=7.2Hz, NAP**CH_3**), 2.72-2.92 (brs, 8H, Aspβ-**CH_2**), 3.91(brs, 4H, NAP**CH_3**O+**CH**), 4.56-4.92(m, 4H, Aspα-**CH**), 7.21-7.72 (m, 6H, NAP**ph-H**).

ESI-MS(*m/z*): calcd. for 691.6 ($[M+H]^+$), obsd. 691.9 Anal. calcd. for $C_{30}H_{34}N_4O_{15}$: C, 52.17, H, 4.96, N, 8.11. found: C, 52.35, H, 4.89, N, 8.02;

Ib: ^{1}HNMR(400MHz, D_2O): δ=1.52(d, 3H, J=7.2Hz NAP**CH_3**), 2.69-2.92(brs, 10H, Aspβ-**CH_2**), 3.91(brs, 4H, NAP**CH_3**O+**CH**), 4.44-4.91(m, 5H, Aspα-**CH**), 7.21-7.72(m, 6H, NAP**ph-H**). ESI-MS(*m/z*): calcd. for 805.7 ($[M]^+$), obsd. 805.1

Ic: ^{1}HNMR(400MHz, D_2O): δ=1.52(d, 3H, J=7.2Hz, NAP**CH_3**), 2.64-2.92(brs, 12H, Aspβ-**CH_2**), 3.91(brs, 4H, NAP**CH_3**O+**CH**), 4.34-4.91(m, 6H, Aspα-**CH**), 7.21-7.72(m, 6H, NAP**ph-H**). ESI-MS(*m/z*): calcd. for 919.8 ($[M-H]^-$), obsd. 919.5

Synthetic Procedure for IIa-c

Compound **11** (0.075g, 0.2mmol) dissolved in anhydrous THF. Activated $Asp_{(4-6)}$ peptide 6a-c (6a, 0.46g, 0.5mmol; 6b, 0.56g, 0.5mmol; 6c; 0.67g, 0.5mmol) and HOBt (0.068g, 0.5mmol) were added to the reaction mixture. DCC (0.103g, 0.5mmol) dissolved in THF was slowly dropped into the solution. After the coupling reaction was completed at room temperature, the solution was filtered twice to remove the DCU. The filtrate was purified by silica column chromatography using $CHCl_2$ and CH_3OH as an eluent, then deprotected the -Obzl with Pd catalyst by hydrogen in CH_3OH at room temperature to afford **IIa-c**. (**IIa**, 0.17g, yield 66%; **IIb**, 0.18g, yield 59%;**IIc**, 0.20g, yield 58%) as white powders.

Data: **IIa**: ^{1}HNMR (400MHz, D_2O): δ=1.48(d, 3H, J=7.2Hz, NAP**CH_3**), 2.60(brs, 4H, **CH_2**CO×2), 2.72-2.99(brs, 16H, Aspβ-**CH_2**), 3.57(brs, 4H, N-**CH_2**×2), 3.98(brs, 4H NAP**CH_3**O+ **CH**), 4.52-4.92(m, 8H, Aspα-**CH**), 7.24-7.80(m, 6H, NAP**ph-H**). ESI-MS(*m/z*): calcd. for 1294.1($[M]^+$), 1317.1($[M+Na]^+$), 648.0($[M+H]^{2+}$), obsd. 1294.8, 1316.6, 648.1 Anal. calcd. for $C_{52}H_{63}N_9O_{30}$: C, 48.26, H, 4.91, N, 9.74 found: C, 48.08, H, 5.01, N, 9.82;

IIb: ^{1}HNMR(400MHz, D_2O): δ=1.47(d, 3H, J=7.2Hz, NAP**CH_3**), 2.58(brs, 4H, **CH_2**CO×2), 2.71-3.04(brs, 20H, Aspβ-**CH_2**), 3.62(brs, 4H, N-**CH_2**×2), 3.97 (brs, 3H, NAP**CH_3**O+**CH**), 4.52-4.92 (m, 10H, Aspα-**CH**), 7.25-7.86 (m, 6H, NAP**ph-H**). ESI-MS(*m/z*): calcd. for 1525.3 ($[M+H]^+$), obsd.1525.5;

IIc: ^{1}HNMR(400MHz, D_2O): δ=1.48(d, 3H, J=7.2Hz NAP**CH_3**), 2.60(brs, 4H, **CH_2**CO×2), 2.74-2.92(brs, 24H, Aspβ-**CH_2**), 3.62(brs, 4H, N-**CH_2**×2), 3.92(brs, 4H, NAP**CH_3**O+**CH**), 4.50-4.94(m, 12H, Aspα-**CH**), 7.27-7.88(m, 6H, NAP**ph-H**). ESI-MS(*m/z*): calcd. for 1778.3 ($[M+Na]^+$), obsd. 1776.9.

Synthetic Procedure for IIIa-c

Compound **16** (0.055g, 0.1mmol)as a core part and DCC (0.08g, 0.4 mmol), HOBt (0.054g, 0.4mmol) were dissolved in anhydrous THF, then the mixture was stirred for 1 h. Activated $Asp_{(4-6)}$ peptide 6a-c (6a, 0.37g, 0.4mmol; 6b, 0.45g, 0.4mmol; 6c; 0.54g, 0.4mmol) was added to the solution under nitrogen atmosphere. After the reaction was completed, DCU was filtered twice and the filtrate was purified with silica column chromatography with $CHCl_2$ and MeOH as an eluent, then deprotected the –Obzl with Pd catalyst by hydrogen in CH_3OH at room temperature to afford **IIIa-c**

(**IIIa**, 0.10g, yield 49%; **IIIb**, 0.10g, yield 43%; **IIc**, 0.11g, yield 42%) as white powders.

Data: **IIIa**: ^{1}HNMR (400MHz, D_2O): δ=1.55(d, 3H, J=6.0Hz NAP**CH_3**), 2.44(brs, 6H, **CH_2**CO×3), 2.77-2.94(br, 24H, Aspβ-**CH_2**), 3.57-3.8 (brs, 12H, O**CH_2**×3+O**CH_2**×3), 3.98(brs, 4H, NAP**CH_3**O+**CH**), 4.60-4.96(m, 12H, Aspα-**CH**), 7.24-7.88 (m, 6H, NAP**ph-H**).

ESI-MS(*m/z*): calcd. for 1928.5 ($[M-H]^-$) 964.3($[M-H]^{2-}$), obsd. 1928.3, 963.9 Anal. calcd. for $C_{75}H_{95}N_{13}O_{47}$: C, 46.66, H, 4.96, N, 9.43. found: C, 46.49, H, 4.81, N, 9.61;

IIIb: ^{1}HNMR (400MHz, D_2O): δ=1.50 (d, 3H, J=6.0Hz NAP**CH_3**), 2.42(brs, 6H, **CH_2**CO×3), 2.77-2.94(br, 30H, Aspβ-**CH_2**×5×3), 3.54-3.82 (brs, 12H, O**CH_2**×3+O**CH_2**×3), 3.98(brs, 4H, NAP**CH_3**O+**CH**), 4.55-4.96(m, 15H, Aspα-**CH**), 7.24-7.88(m, 6H, NAP**ph-H**).

ESI-MS(*m/z*): calcd. for 2276.9 ($[M+H]^+$), obsd. 2276.6;

IIIc: ^{1}HNMR (400MHz, D_2O): δ=1.56(d, 3H, J=6.0Hz NAP**CH_3**), 2.45(brs, 6H, **CH_2**CO×3), 2.70-2.99(brs, 36H, Aspβ-**CH_2**), 3.69 (brs, 12H, O**CH_2**×3+O**CH_2**×3), 3.99(brs, 4H, NAP**CH_3**O+**CH**), 4.55-4.96(m, 18H, Aspα-**CH**), 7.26-7.89(m, 6H, NAP**ph-H**).

ESI-MS(*m/z*): calcd. for 2620.7 ($[M+H]^+$), obsd. 2620.7

In Vitro HAP Binding Assay

Conjugates were dissolved in PBS(pH7.4, 5ml, concentration 100 μg/ml) and incubated with HAP(10mg) in a shaker bath at 37°C. Each formulation was cultured for 0.5h and 16h. After the prescribed time, conjugate solutions were removed from the water bath and centrifuged for 2 min at 2500rpm. Standard conjugate solutions were also prepared from 16μg/ml to 400μg/ml to generate a concentration calibration curve. Concentrations of non-bound conjugates were determined by UV spectrophotometry absorbance at 273nm. A control was tested in Ca^{2+} containing solution to corroborate the HAP binding further. The result was shown in Figs. (**2** and **3**).

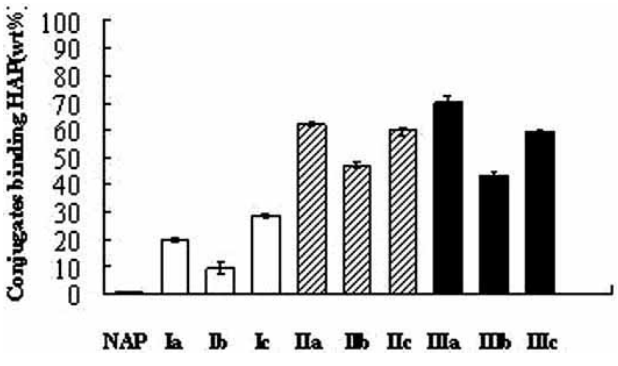


Fig. (2). Percentage of conjugates bound to HAP at 0.5h.Error bars represent the mean and standard deviation of three independent experiment.

After that assay, we chose three conjugates (the monomer, dimer and trimer) which displayed a good HAP affinity and determined 0.5, 1, 2, 4, 8, 16 h' HAP binding with the same method respectively. The result was shown in Fig. (**4**).

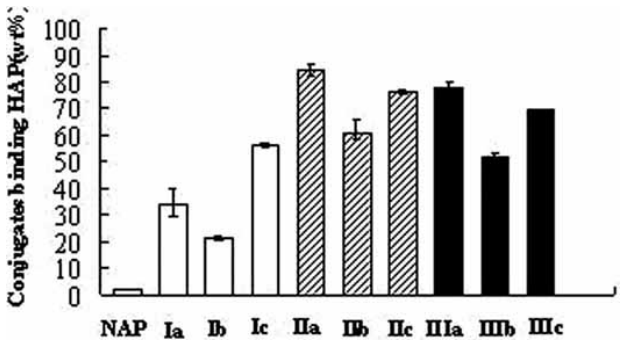


Fig. (3). Percentage of conjugates bound to HAP at 16h.Error bars represent the mean and standard deviation of three independent experiment.

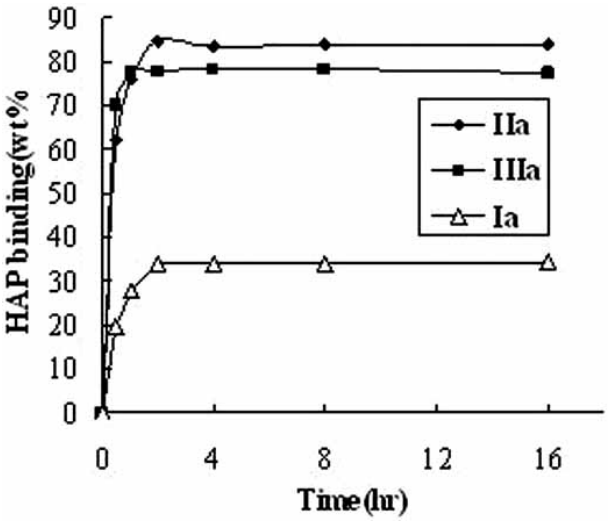


Fig. (4). Change of percent conjugates Ia, IIa, IIIa bound to HAP.

RESULTS AND DISCUSSION

We designed and synthesized Naproxen-Peptide-Monomer and Naproxen-Peptide-Dendrimer as bone targeting prodrugs by a conventional coupling strategy. All modified naproxen conjugates exhibited some binding to HAP *in vitro* and the naproxen-peptide-dendrimers showed a more greater binding rate(up to 90%)than the monomers after 16h. Unmodified naproxen showed no greater than 2% mean binding.

In the synthesis of the naproxen peptide dendrimers, using IBCF/NMM was more difficult to achieve than previous amidation reactions. To overcome the problem of steric hindrance, several other coupling reagents(DCC, CDI, EDCI, etc.) were used and we found DCC/Hobt method afforded the product in a relatively high yield.

The high degree of symmetry in these dendrimers enabled facile confirmation of both structure and purity by NMR techniques. For example in the ^{1}HNMR spectrum of dendrimer III, the core protons observed the resonance signals at 1.44 (s), 2.45 (brs), and 3.69 (brs) ppm were clearly distinguishable from the resonances arising from the periph-

ery (Asp oligopeptides) at 2.71 (m) and 4.84 (m) ppm. Integration of the respective areas of the core protons and β-methine protons of Asp oligopeptides confirmed the complete coupling of the central cores and peptides 6. Furthermore, the structures of these dendrimers were further verified by ESI MS and GPC. The GPC results were outlined in Table **1**. All of the spectra displayed a very prominent peak corresponding to the dendrimers complexed with protons or sodium cation. Moreover, elemental analysis was also in good agreement with those of the signed structures.

Table 1. Molecular Weights of Synthesized Dendrimers Measured by GPC

Compound	RetTime[a]	Mw[a]	Mw/Mn[a]	*m/z*[b]
Ia	17.045	1233	1.05	691
IIa	16.490	2728	1.38	1294
IIIa	16.238	3318	1.18	1928

[a]Measured by GPC (polystyrene standards) with THF as an eluent.
[b]Measured by ESI mass spectroscopy.

The monomer naproxen-Asp_{4-6} exhibited some HAP affinity and the Asp_6 conjugate displayed the best HAP binding. Comparing with the binding results of bisphosphonate compounds in our lab [14], the binding rates of Asp oligopeptides were routine. This study revealed the HAP binding properties of these peptides.

Comparing the HAP affinitiy of the naproxen-peptide monomer with the naproxen-peptide dendrimer. According to the result shown in Figs. (**2** and **3**), it was observed that a dendritic approach was successful. Both conjugates with two or three $Asp_{(4-6)}$ fragments showed a greater HAP affinity than monomers and all naproxen-peptide dendrimers demonstrated a rapid adsorption. The trimer's binding rate was litte less than the dimer's because the steric hindrance played an important role [15].

Fig. (**4**) described that the dimer and trimer exhibited greater than 80% binding rate and approached their respective binding plateaus by two hours. The precise mechanism for the more rapid binding of the trimer over the other ones was not yet understood, but it could be a result of the larger domain of negative charges to chelate with calcium in the HAP.

CONCLUSIONS

In summary, the naproxen-peptide monomer and the naproxen-peptide dendrimer as bone targeting prodrugs were readily synthesized and characterized. Both prodrugs bound to HAP, a model for bone. The peptide dendrimers with higher numbers of total poly(aspartic acid) groups attached per naproxen than naproxen-peptide monomer exhibited their intended effect, faster initial binding and greater total binding rates. Although the water solubility of naproxen was poor, these prodrugs showed a good solubility in water. Therefore, based on the *in vitro* data presented here, we suggested that the Asp peptide-dendrimer had potential for drug delivery to bone. We are currently exploring higher generations of dendritic molecules bearing more Asp_n sequences and developing a suitable animal model for *in vivo* evaluation.

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