Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 621–624

Design and synthesis of a peptide-PEG transporter tool for carrying adenovirus vector into cells☆

Mitsuko Maeda,[a] Shinya Kida,[a] Keiko Hojo,[a] Yusuke Eto,[b] Jian-Qing Gaob,[b] Shinnosuke Kurachi,[b] Fumiko Sekiguchi,[b] Hiroyuki Mizuguchi,[c] Takao Hayakawa,[d] Tadanori Mayumi,[a] Shinsaku Nakagawa[b] and Koichi Kawasaki[a,*]

[a]*Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Nishi-ku, Kobe 651-2180, Japan*
[b]*Graduate School of Pharmaceutical Sciences, Osaka University, Yamadaoka, Suita 565-0871, Japan*
[c]*Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan*
[d]*National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan*

Received 17 September 2004; accepted 16 November 2004
Available online 23 December 2004

Abstract—The adenovirus vector is a promising carrier for the efficient transfer of genes into cells via the coxackie-adenovirus receptor (CAR) and integrins (αvβ3 and αvβ5). The clinical use of the adenovirus vector remains problematic however. Successful administration of this vector is associated with side effects because antibodies to this vector are commonly found throughout the human body. To make the adenovirus vector practicable for clinical use, it is necessary to design an auxiliary transporter. The present study describes the use of Arg-Gly-Asp(RGD)-related peptide, a peptide that binds to integrins, as an auxiliary transporter to aid efficient transport of adenovirus vector. Furthermore, poly(ethylene glycol) (PEG) was also used as a tool to modify the adenovirus such that the risk of side effects incurred during clinical application was reduced. The present study describes the design, preparation and use of (acetyl-Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro-$_{\beta}$Ala)$_2$Lys-PEG-$_{\beta}$Ala-Cys-NH_2[(Ac-YGGRGDTP$_{\beta}$A)$_2$K-PEG-$_{\beta}$AC] as an efficient peptide-PEG transporter tool for carrying adenovirus vector into cells. (Ac-YGGRGDTP$_{\beta}$A)$_2$K-PEG-$_{\beta}$AC was coupled with 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester and the resulting 6-[(Ac-YGGRGDTP$_{\beta}$A)$_2$K-PEG-$_{\beta}$AC-succinimido]hexanoic acid *N*-hydroxysuccinimide ester reacted with adenovirus. The modified adenovirus with the peptide-PEG hybrid exhibited high gene expression even in a CAR-negative cell line, DC2.4.

Gene therapy is a new field of clinical treatment for intractable diseases. A key aspect of gene therapy, and a major determinant of its success, lies in the vector used for transgenesis. Adenovirus vectors (Ad) are widely used as vectors for gene therapy experiments[2] since they exhibit highly efficient transduction and gene expression. Ad infection is performed in two steps; firstly Ad binds to its receptor, coxackie-adenovirus receptor (CAR),[3] followed by receptor-mediated endocytosis via αvβ3 and αvβ5 integrins.[4] Both of these integrins are known as a receptor of peptides containing the Arg-Gly-Asp (RGD) sequence. Ad is able to transfer genes efficiently into both dividing and nondividing cells, but some problems remain in terms of its clinical application. Side effects are common because antibodies to Ad are commonly found within the human body (Fig. 1).[5]

Poly(ethylene glycol) (PEG) is a low toxicity polymer and its hybrid formation (conjugation) with a protein is a method known to improve certain characteristics of the chosen protein (such as response to an antibody, response to enzymatic degradation, solubility to aqueous and organic solvents and prolongation of biological activities). As a result of this new methodology, the formation of a protein-PEG hybrid has become known as 'pegylation', a term that is now commonly used. Several studies have investigated the pegylation of Ad;[6] results demonstrated that pegylated Ad exhibited enhanced circulation and half-life in blood depending on the rate of pegylation. Transduction by the pegylated Ad was not disturbed in the presence of its antibody.[7] However the ability of the pegylated Ad to penetrate into cells

Keywords: Adenovirus vector; RGD; Poly(ethylene glycol); Transduction; Peptide synthesis.
☆See Ref. 1.
*Corresponding author. Tel.: +81 78 974 4794; fax: +81 78 974 5689; e-mail: kawasaki@pharm.kobegakuin.ac.jp

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.11.055

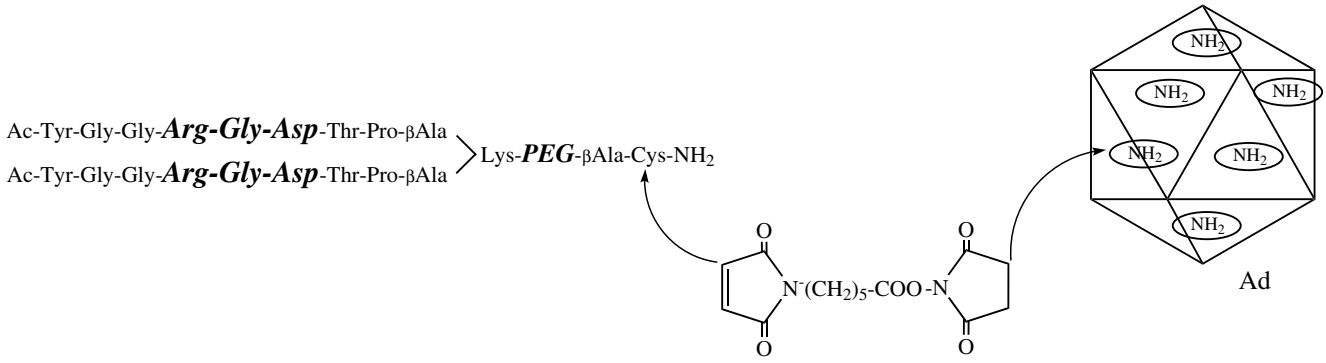

Figure 1. Design of an efficient peptide-PEG transporter tool for carrying adenovirus vector into cells via integrins.

through the coxackie-adenovirus receptor (CAR) was reduced by the steric hindrance of the associated PEG chains. To overcome this problem, an Arg-Gly-Asp(RGD)-related peptide, Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro (YGGRGDTP),[8] was considered as a tool to allow Ad to penetrate into cells via its receptors (αvβ3 and αvβ5 integrins). The peptide was reported to show good endocytotic ability and RGD sequence in the peptide was reported to be necessary to exhibit this activity. Since the final synthetic product will be reacted with Ad by the active ester method, the RGD-related peptide should not have side chains, which will be acylated by the active ester method. YGGRGDTP has no such side chain (Fig. 2).

In an attempt to ensure efficient affinity between the peptide and integrins, a bivalent peptide derivative through Lys (K) was designed (Fig. 1). In order to prepare a hybrid of the peptide and PEG, an amino acid type PEG (aaPEG) was utilized. To introduce the peptide-PEG hybrid to Ad, a heterofunctional cross-linking reagent with amine and sulfhydryl reactivity, 6-maleim-

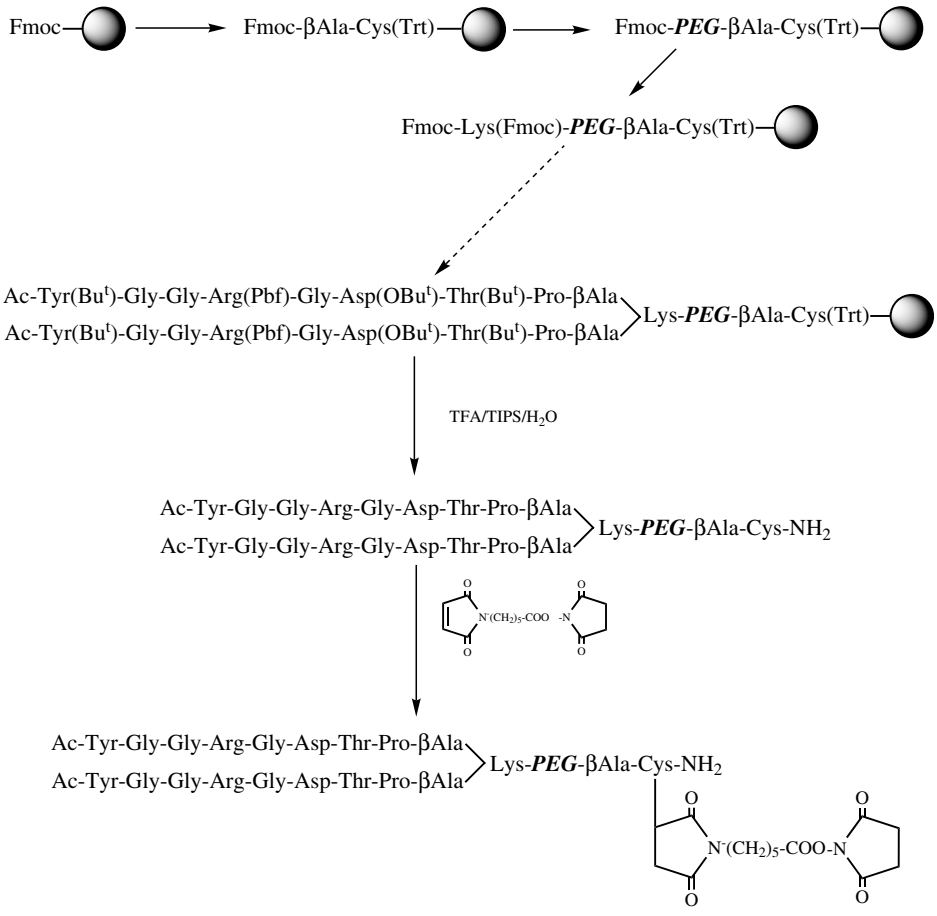

Figure 2. Synthetic scheme for the preparation of the PEG-(RGD-peptide) hybrid used to carry Ad into cells.

Table 1. Synthetic protocol for the solid-phase synthesis of Ac-YGGRGDTP)$_2$K-PEG-$_\beta$AC

Step	Reagent	Volume	Period	Time
1	20% Piperidine/DMF	5 mL	5 min	1
		5 mL	0.5–1 h	1
2	DMF (wash)	5 mL	3 min	5
3	Fmoc-amino acid derivative	0.75 mmol		
	0.5 M HBTU/HOBt/DMF	1.5 mL	2–4 h	
	2 M DIEA/NMP	0.75 mL		
4	DMF (wash)	5 mL	3 min	5

idohexanoic acid *N*-hydroxysuccinimide ester[9] (MHS), was utilized. As a result, Cys was also incorporated in the hybrid. βAla ($_\beta$A) was also incorporated into the hybrid as a spacer. The final hybrid, (acetyl-Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro-$_\beta$Ala)$_2$Lys-PEG-$_\beta$Ala-Cys-NH_2, (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC amide, was thus designed (Fig. 2). The hybrid was synthesized by manual solid-phase methodology using fluorenylmethoxycarbonylamino acids (Fmoc-amino acids) on Rink amide resin (0.67 mequiv L/g, PE BioSystems. 370 mg, 0.25 mmol)[10] according to the protocol shown in Table 1. The following amino acids were purchased from Watanabe Chemical Industry Ltd (Japan) and Peptide Institute Inc. (Japan); Fmoc-Arg(Pbf)-OH (Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Fmoc-Cys(Trt)-OH (Trt: trityl), Fmoc-Tyr(Bu^t)-OH (Bu^t: *tert*-butyl), Fmoc-Thr(Bu^t)-OH, Fmoc-Asp(OBu^t)-OH, N^α,N^ε-diFmoc-Lys-OH, Fmoc-Gly-OH, Fmoc-$_\beta$Ala-OH and Fmoc-Pro-OH. Fmoc-aaPEG-OSu (–OSu: *N*-hydroxysuccinimide ester) (MW 3400) was purchased from Shearwater polymers Inc. and MHS was purchased from Dojindo Laboratories, Japan). All of the above materials were used as supplied without any further purification. As described in the protocol (Table 1), Fmoc groups were removed by 20% piperidine/dimethylformamide (DMF) treatment and coupling reactions were performed with 0.5 M 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/DMF in the presence of 1-hydroxybenzotriazole (HOBt) in *N*-methylpyrrolidinone (NMP). Since Fmoc-aaPEG-OSu did not react with $_\beta$Ala-Cys(Trt)-Rink amide resin, HBTU, HOBt and diisopropylethylamine (DIEA) were added to the reaction mixture. The reaction did not proceed without the addition of HBTU, HOBt and DIEA. Since the deprotection reaction of the Fmoc group decreased in rate after the introduction of Fmoc-aaPEG, the following deprotection procedure was performed for 1 h. After the introduction of Lys, the amount of each added reagent was doubled, and each step involving the introduction of Fmoc-amino acid was performed using a double coupling reaction. The amino group of the *N*-terminal Tyr was acetylated with acetic anhydride. The synthetic (Ac-Tyr(Bu^t)-Gly-Gly-Arg(Pbf)-Gly-Asp-(OBu^t)-Thr(Bu^t)-Pro-$_\beta$Ala)$_2$Lys-aaPEG-$_\beta$Ala-Cys(Trt)-Rink amide resin was then treated with a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIPS) (95:2.5:2.5), and the resulting crude peptide-PEG hybrid, (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC, 462 mg), was purified by HPLC (Fig. 3). The purified hybrid (55 mg, 10 mmol)[11] dissolved in PBS (pH 7.4, 1 mL), and the

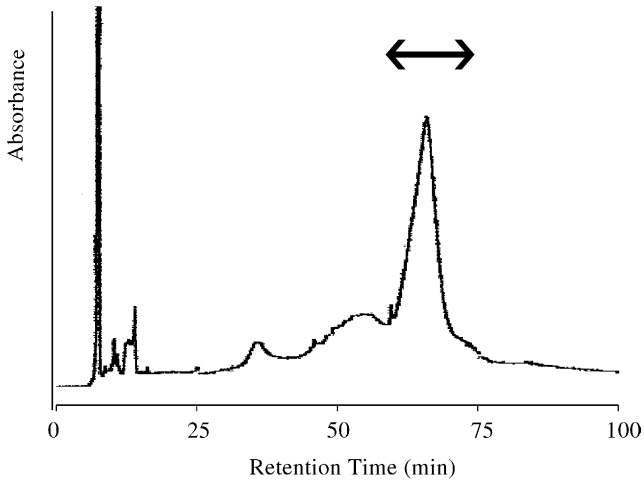

Figure 3. HPLC profile of synthetic crude (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-SHS. Column: DAISOPAK SP-120-5-ODS-B (20 × 250 mm). Flow rate: 10 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 10:90 → 70:30 (60 min). OD at 220 nm.

heterofunctional cross-linkage reagent (MHS 3.1 mg, 10 mmol) dissolved in dimethylsulfoxide (DMSO, 0.1 mL), were combined and the mixture stirred for 0.5 h. Since the product of this reaction, [(Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-SHS)] (SHS: 6-succimidohexanoic acid *N*-hydroxysuccinimide ester) was easily hydrolyzed in water, the reaction mixture was frozen immediately and kept in a freezer to await the next reaction step. Purification of the reaction product by HPLC was attempted, but was not successful since the *N*-hydroxysuccinimide ester portion of the product hydrolysed easily in the presence of water. We observed that 50% of MHS was hydrolyzed at pH 7.4 after 1.5 h at room temperature (Fig. 4). Ad, which has luciferase expression ability, was modified with (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-SHS solution at 37 °C for 45 min with gentle stirring and the transduction efficiency of the resulting modified Ad (RGDpep-PEG-Ad) via receptor-mediated endocytosis was examined with A549 (CAR + and integrins-positive) and DC2.4 (CAR − and integrins-positive) cell lines using a Luciferase Assay System Kit (Promege, USA) and a Microlumat Plus LB 96 instrument (Perkin–Elmer, USA), after

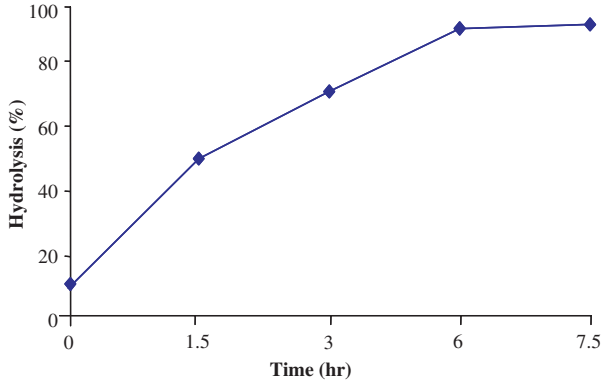

Figure 4. Hydrolysis of 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHS) in water at pH 7.4.

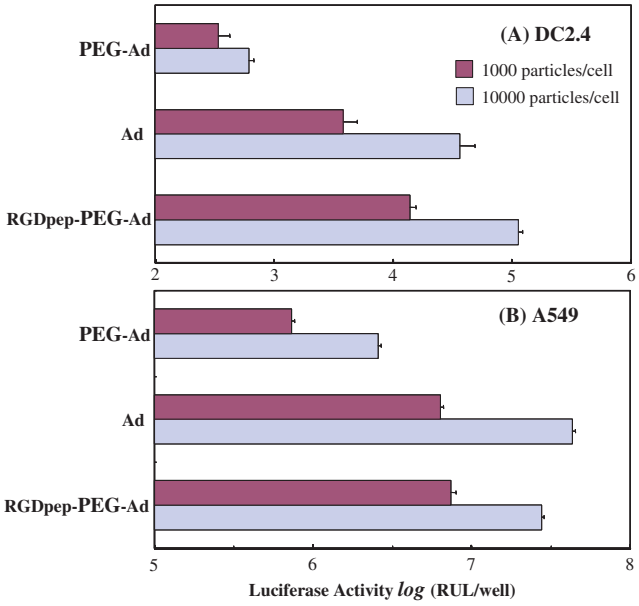

Figure 5. Transduction efficiency of RGDpep-PEG-Ad into DC2.4 (CAR −) cells and A549 (CAR +) cells. (A) DC2.4 cells (2×10^{10} cells) and (B) A549 cells (2×10^{10} cells) were transduced with 1000 and 10,000 particles/cells of Ad, PEG-Ad and RGDpep-PEG-Ad, respectively. Luciferase expression was measured after 24 h. Each point represents mean ± SD ($n = 3$).

cells were first lysed with Luciferase Cell Culture Lysis Reagent (Promega, USA). Luciferase activity was described as relative light unit per well (RLU/well). We thus measured the luciferase activity of cells transducted with Ad, PEG-Ad[7] RGDpep-PEG-Ad, as shown in Figure 5. A549 cells that had been infected with Ad or RGDpep-PEG-Ad exhibited luciferase activity in similar proportions. On the other hand, the luciferase activity of DC2.4 cells that had been infected with Ad was much lower than that of DC2.4 cells that had been infected with RGDpep-PEG-Ad. Furthermore, infection of cells with RGDpep-PEG-Ad was not blocked in the presence of an antibody to Ad (data is not shown). These results indicate that RGDpep-PEG-Ad could be transducted into cells via the integrins and was protected from the antibody by its pegylated structure.

In summary, we designed and prepared (Ac-YGGRGDTP$_{\beta}$A)$_2$K-PEG-$_{\beta}$AC as an efficient auxiliary transporter tool for carrying Ad into cells. Although (Ac-YGGRGDTP$_{\beta}$A)$_2$K-PEG-$_{\beta}$AC-SHS could not be purified by HPLC owing to its instability in water, quality of this tool when constructed in situ was still sufficient to modify Ad. Various active esters of 6-maleimidohexanoic acid are presently being examined in order to obtain a stable 6-[(Ac-YGGRGDTP$_{\beta}$A)$_2$K-PEG-$_{\beta}$AC-succinimido]hexanoic acid active ester in water.

Recently, Ogawara et al.[12] reported a procedure that modified Ad in two steps, using PEG and cyclic RGD peptide. These authors prepared PEG-Ad and then combined cyclic RGD peptide (Ansynth, Netherlands) with the pegylated Ad. Our own future studies will investigate peptide-PEG transporters, which can modify Ad in just one step.

References and notes

1. This paper was reported at The 123rd Annual Meeting of The Pharmaceutical Society of Japan, Nagasaki, Japan, Mar 27–29, 2003.
2. (a) Crystal, R. G. *Science* **1955**, *270*, 404–4010; (b) Wilson, J. M. *New Engl. J. Med.* **1996**, *334*, 1185.
3. (a) Bergelson, J. M.; Cunningham, J. A.; Droguett, G.; Kurt-Jones, E. A.; Krithivas, A.; Hong, J. S.; Horwitz, M. S.; Crowell, R. L.; Finberg, R. W. *Science* **1997**, *275*, 1320; (b) Bewley, M. C.; Springer, K.; Zhang, Y. B.; Freimuth, P.; Flanagan, J. M. *Science* **1999**, *286*, 1579.
4. (a) Wickham, T. J.; Mathias, P.; Cheresh, D. A.; Nemerow, G. R. *Cell* **1993**, *73*, 309–319; (b) Bai, M.; Harfe, B.; Freimuth, P. *J. Virol.* **1993**, *67*, 5198.
5. Van Ginkel, F. W.; Liu, C.-G.; Simecka, J. W.; Dong, J.-Y.; Greenway, T.; Frizzell, R. A.; Kiyono, H.; McGhee, J. R.; Pascual, D. W. *Hum. Gene Ther.* **1995**, *6*, 895.
6. (a) Romanczuk, H.; Galer, C. D.; Zabner, J.; Barsomian, G.; Wadsworth, S. C.; O'Riordan, C. R. *Hum. Gene Ther.* **1999**, *1*, 2615; (b) Croyle, M. A.; Chirmule, N.; Zhang, Y.; Wilson, J. M. *J. Viol.* **2001**, *75*, 4792; (c) Croyle, M. A.; Chirmule, N.; Zhang, Y.; Wilson, J. M. *Hum. Gene Ther.* **2002**, *10*, 1887.
7. Eto, Y.; Gao, J.; Sekiguchi, F.; Kurachi, S.; Katayama, K.; Mizuguchi, H.; Hayakawa, T.; Tsutsumi, Y.; Mayumi, T.; Nakagawa, S. *Biol. Pharm. Bull.* **2004**, *27*, 936.
8. Erbacher, P.; Remy, J. S.; Behr, J. P. *Gene Ther.* **1999**, *6*, 138.
9. (a) Hashida, S.; Imagawa, M.; Inoue, S.; Ruan, K. H.; Ishikawa, E. *J. Appl. Biochem.* **1984**, *6*, 53; (b) Fargeas, C.; Hommel, M.; Maingon, R.; Dourado, C.; Monsigny, M.; Mayer, R. *J. Clin. Microbiol.* **1996**, *2*, 241.
10. (a) Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787; (b) Bernatowicz, M. S.; Daniels, S. B.; Köster, H. *Tetrahedron Lett.* **1989**, *30*, 4645.
11. Yield 78 mg (6%, calculated from NH_2 content of the used resin). Amino acid ratios in an acid hydrolysate: Asp, 0.80; Gly, 3.21; Arg, 0.87; Thr, 1.06; Pro, 1.02; Tyr, 0.91; Lys, 0.50. Peptide content calculated from the amino acid analysis, 0.15 mmol/g.
12. Ogawara, K.; Rots, M. G.; Kok, R. J.; Moorlag, H. E.; Loenen, A. V.; Meijer, D. K. F.; Haisma, H. J.; Molema, G. *Human Gene Ther.* **2004**, *15*, 433.