Protein kinase Cα : its potential for cancer-specific gene delivery and regulaiton of cancer drug resistance

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Protein Kinase Cα: Its Potential for Cancer-Specific Gene Delivery and Regulation of Cancer Drug Resistance

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Abstract

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Intracellular signal transduction has prominent roles for many cellular processes in living cells, which is generally modulated by a large number of protein kinases and phosphatases regulating diverse functions, such as proliferation and survival. Because of their critical roles in cellular processes, aberrant activities of intracellular signaling are known to be involved in many diseases. The main objective of this thesis is to focus on abnormally activated intracellular signal, protein kinase Cα (PKCα), as an attractive target, intimately implicated in many cancers and abnormal proliferation and differentiation in transformed cell lines.

Here, I investigate the development of specific and stable gene delivery systems responding to abnormally activated PKCα in cancers, presenting two approaches, using of cationic polymer backbone and introducing of hydrophobic interaction. These cellular signal-responsive systems exhibited quite high transgene expression responding to abnormally activated PKCα, and presented the effective cellular uptake and capable of endosomal escape of polyplexes caused by a strong pH buffering capacity of PEI units at around 5-6 in endosome causing the so-called proton sponge effect, consistent with their efficient transgene expression both in vitro and in vivo. These approaches are able to be served as an alternative application for cancer-specific gene therapy.

Moreover, I focus on PKCα as an attractive modulator for successful cancer chemotherapy, in which hyperactivated in drug-resistance cancer cells, and propose one potential for reversal of drug resistance by regulating this activated PKCα. The effective inhibition of PKCα activity presented increased drug accumulation in drug-resistant MCF-7/ADR cells, providing evidence that PKCα has a prominent role in the regulation of drug efflux. This approach may improve response of therapeutic drug, and consequently contribute to chemotherapeutic efficacy in drug-resistance cancers.

Contents

CHAPTER 1 Introduction

All living organisms are composed of one or more living cells, which are the fundamental units of life. These cells produce various cellular signals regulating a diversity of function, such as proliferation and survival, for their mutual requirements. All the information is processed by these signals to form appropriate cellular responses. In this process, many enzymes change their activities and such enzymatic reactions make complicated network system to crosstalk each other. This sophisticate enzymatic network is called 'cellular signal transduction system'. Although this system was mainly an objective of basic cellular biology, it has now received increased attention for medical and pharmaceutical research since the relationship between abnormality and numerous diseases has been clarified in these days.

In this thesis, I focus on 'intracellular signal transduction' that is aberrantly activated in many cancers and describe new approaches that aim to introduce intracellular signal-responsive transgene regulation systems as an alternative application for cancer-specific gene delivery, and suggest potential for reversal of drug resistance by intracellular regulation of signal transduction. This chapter presents background information that is integral to the understanding of the unique approaches in this research.

1.1 Intracellular signaling pathways

Signal transduction enables cells to regulate the behaviors in its requirements. To communicate with other cells, external signals, such as hormone, growth factors, cytokines or chemokines are used. These signaling are mainly received by receptors, which can selectively recognize specific molecules on a plasma membrane, and then conveyed into the target cell interiors via intracellular signaling pathways (**Figure 1.1**).[1] The resulting intracellular signaling process is propagated from the cell membrane to the nucleus or other distant targets through the response of sequential signaling. This signal transduction is mainly regulated through protein kinases and phosphatases with their tight and reversible control on protein phosphorylation.[2-6]

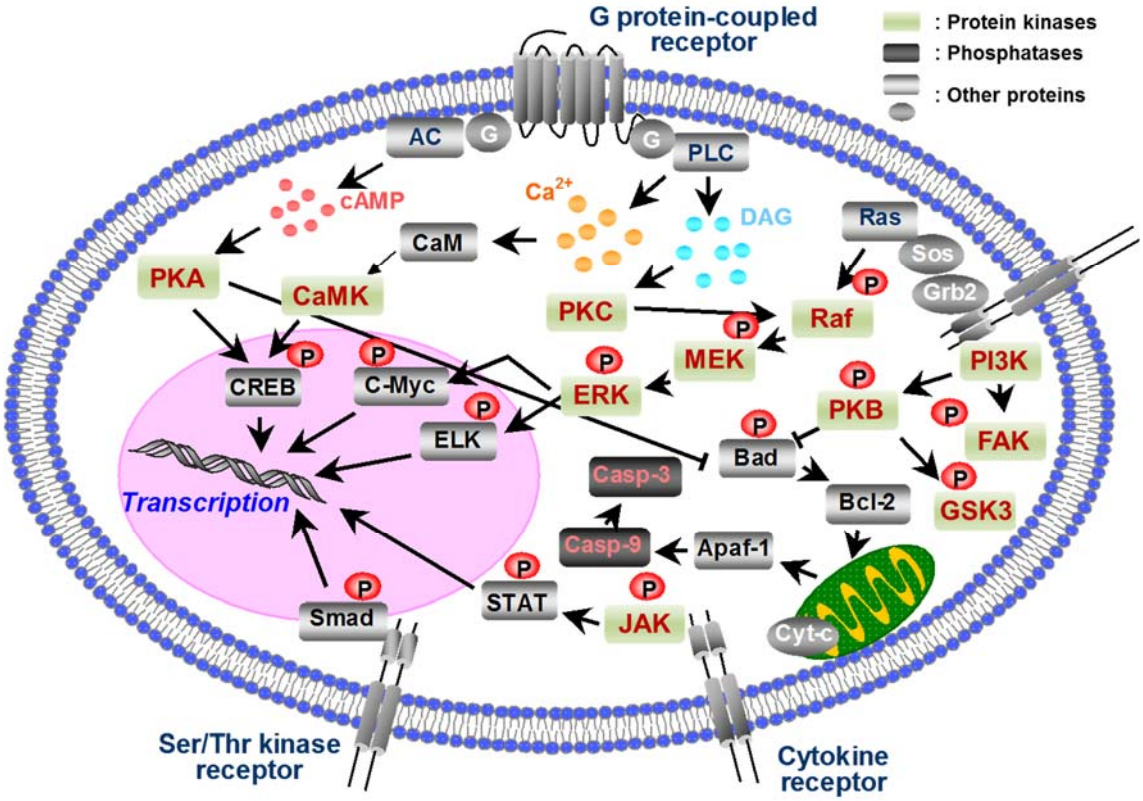


Figure 1.1. Simplified scheme of intracellular signaling pathways. Cellular processes, such as proliferation and survival, are generally modulated by the modifications of the structure and function of cellular proteins, which is triggered by extracellular signaling through membrane receptors, such as ion channel linked receptors, enzyme-linked receptors, and G protein-coupled receptors. It has been implicated mostly in phosphorylation and dephosphorylation by a variety of protein kinases and phosphatases.

Protein kinases consist of more than 500 kinds of families and have critical roles of intracellular signaling pathways. These protein kinases catalyse the transfer of the terminal γ-phosphate from ATP to a serine, threonine, or tyrosine residue in specific site in substrate proteins (**Figure 1.2**).[7] Phosphorylation of protein can control its enzymatic activity, its interaction with other proteins and molecules, and its location in the cell. These are well regulated by sequential activities of protein kinases in many cellular processes, including metabolism, cell growth, differentiation, and apoptosis.[8-13] Because of a critical role of protein kinases in cellular activities, modulation of protein phosphorylation is identified to understand a mechanism of cellular events in normal

cells. While, abnormal activities of such protein kinases are known to be involved in many diseases, including cancer, diabetes, and inflammation. Thus, many approaches have been studied to define mutations and dysregulation of protein kinases in physiological and phathological situations.[14-16]

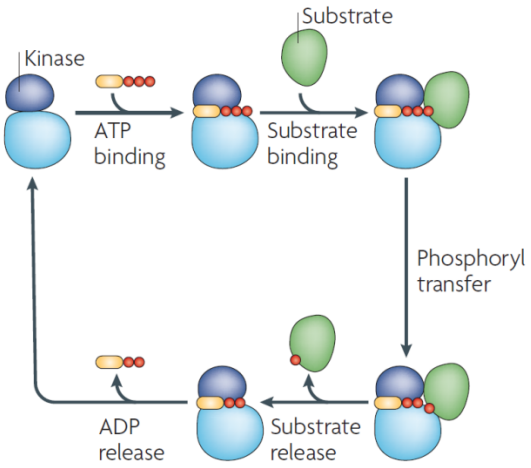


Figure 1.2. The basic catalytic cycle for substrate phosphorylation by a kinase.

1.2 Protein kinase Cα (PKCα) as an attractive target enzyme in cancer

As mentioned above, protein kinases have the significant roles in intracellular signal transduction pathways by their revisable control with phosphatases on protein phosphorylation. However, misregulation of kinase function has become apparent in many diseases. Especially, a large number of protein kinases have been found to be intimately involved in the processes to tumor cell proliferation and survival (**Table 1.1**).[17-22] Thus, these protein kinases can be considered as a potential therapeutic target for the treatment of cancer formation and progression in human pathology.

Among a number of protein kinases, protein kinase C (PKC), a phospholipid-dependent Ser/Thr kinase, appears to be involved in intracellular signaling response to many hormones and growth factors for cell proliferation, differentiation, apoptosis, and angiogenesis. PKCs are classified into three subfamilies, based on structural and activation characteristics: conventional or classic PKCs (cPKCs; α, βI, βII, and γ) containing a diacylglycerol (DAG)/phorbol ester-binding C1 domain and a Ca^{2+}-binding

C2 domain, novel or non-classic PKCs (nPKCs; δ, ε, η, and θ) containing C1 domains, and atypical PKCs (aPKC; ζ, ι, and λ) containing an atypical C1 domain, which are regulated independently of Ca^{2+} or DAG (**Figure 1.3**).[23-29]

Table 1.1. Genetic mutation or translocation lists of protein kinases in cancer.[22]

	Kinase	Tumor / Cancer Types
RTK	EGFR	Breast, lung, glioma
	HER-2/ErbB2	Breast, ovarian, colon, lung, gastric
	IGF-IR	Colorectal, pancreatic, breast, ovarian, MM
	PDGFR-α	Glioma, glioblastoma,ovarian, HES
	PDGFR-β	CMML, glioma, DFSP
	c-Kit	GIST, seminoma, mastocytosis
	Flt-4, Flt3	AML
	FGFR1	CML, Stem cell myeloproliferative disorder
	FGFR3	Multiple myeloma
	FGFR4	Breast, ovary
	c-Met	Glioblastoma, Colorectal, Hepatocellular carcinoma, renal carcinoma, HNSCC metastases
	RON	Colon, hepatocellular carcinoma
	c-Ret	Thyroid carcinoma, MEN2A, MEN2B, FTMC familial & sporadic
	ALK	Anaplastic large cell lymphoma, lung, neuroblastoma
CTK	c-SRC	Lung, colon, breast & prostate
	c-YES	Lung, colon, breast & prostate
	Abl	CML
	JAK-2	CML, T-ALL, solid
S/T Kinase	Akt	Multiple
	ATM	Ataxia telangiectasia
	Aurora A & B	Multiple
	CDKs	Multiple
	mTOR	Multiple
	PKCi	Non-small cell lung, ovarian
	PLKs	Multiple
	b-Raf	Colon, thyroid, melanoma
	S6K	Multiple
	STK11/LKB1	Peutz-Jeghers syndrome
LK	PI3K	Prostate, colorectal, breast
	SK1	Breast, prostate

* RTK: receptor tyrosine kinase, CTK: cytoplasmic tyrosine kinase, S/T Kinase: serine/threonine kinase, LK: lipid kinase

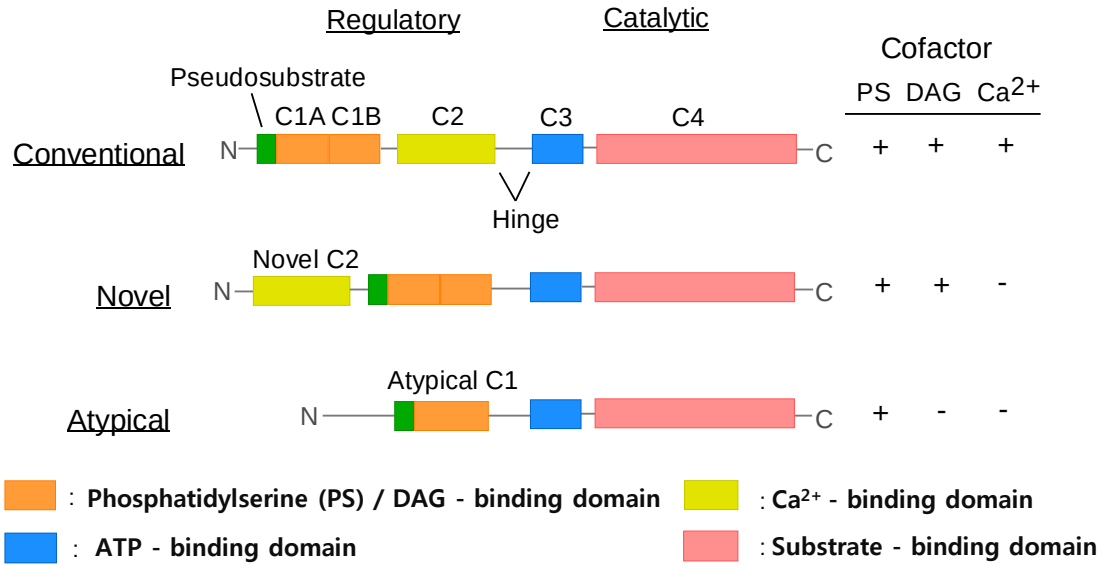


Figure 1.3. Structure of protein kinase C family members showing domain composition and activators.[29]

Activation of PKC isoforms is connected with the two main signaling molecules, DAG and inositol triphosphate (IP_3). Binding of a ligand to a G protein-coupled receptor or receptor tyrosine kinase (RTK), $PI(4,5)P_2$, which is generated by stepwise phosphorylation of phosphatidylinositol (PI), is converted into DAG and IP_3 by phospholipase C (PLC) at the membrane of cells. The induced DAG activates both cPKCs and nPKCs, and the efflux of Ca^{2+} from endoplasmic reticulum (ER) is generated by the induced IP_3 as the cofactors of cPKCs activation. Finally the activated PKC induces the phosphorylation of its specific substrate (**Figure 1.4**).[27,30-32]

Despite the significant roles of PKCs in cellular processes, PKC isoforms have been implicated in cancer formation and progression. For example, the signaling abnormalities of PKCs contribute to malignancy and also consider as a potential therapeutic target in various cancer as follows.[33-36]

- Skin cancer : PKCα, PKCβI, PKCζ, PKCδ, PKCε [37-41]
- Colon and gastric cancer : PKCα, PKCδ, PKCβII, PKCι [42-45]
- Prostate cancer : PKCα, PKCδ, PKCε [36,45-49]
- Ovarian, breast, and endometrial cancer : PKCι, PKCα, PKCζ [50-53]
- Lung cancer : PKCι, PKCζ, PKCδ, PKCε, PKCα [54-61]
- Brain tumors : PKCδ, PKCζ, PKCε, PKCη, PKCγ [62-69]
- Multiple, leukemias, and lymphomas : PKCδ, PKCι, PKCζ, PKCβ, PKCγ [70-76]

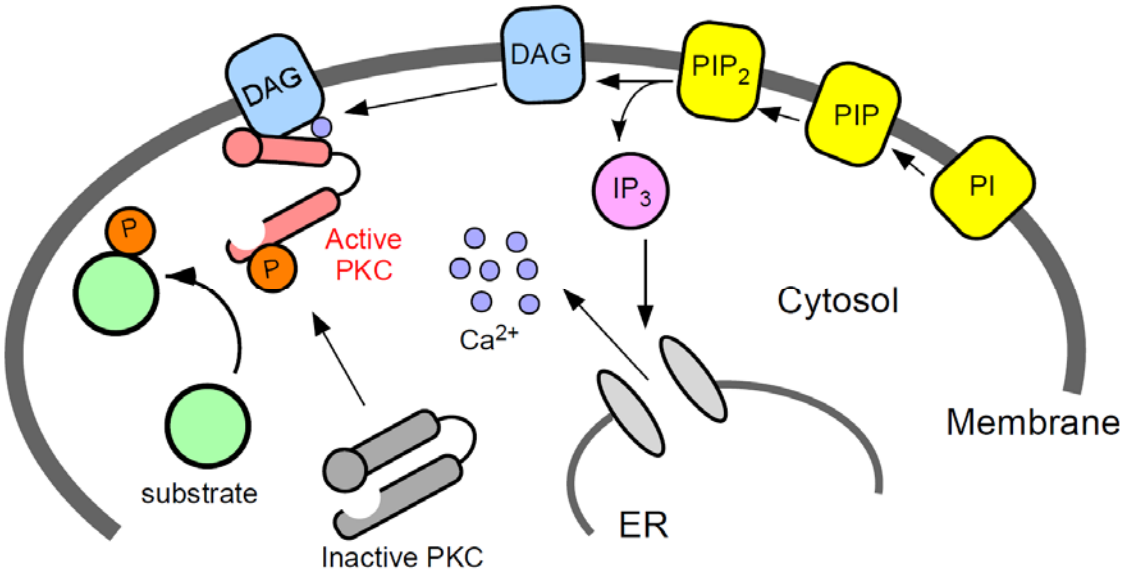


Figure 1.4. Activation of PKCs is regulated by DAG and IP_3 from cell membrane phospholipids.[27]

Among these PKC isoforms, PKCα is multifunctional kinase which is ubiquitously expressed in many tissues with its biological function, such as proliferation, apoptosis, differentiation, cell migration / adhesion, cardiac hypertrophy / angiogenesis, and inflammation. However, abnormal levels of PKCα activity have been identified in many types of tumors and has been clarified that the activity is closely related to cancer malignancy, bad prognosis and metastatic activity.[26,45,77-79] In addition, this PKCα activity is highly implicated with multidrug resistance (MDR) of cancer cells, which is a major factor in a failure of cancer chemotherapy.[80-82] Therefore, selective targeting and modulating of PKCα has potential for the treatment of cancer and contributes to emerge prospective approaches in human pathology.

1.3 Intracellular signal-responsive transgene regulation system

Intracellular signal transduction has essential roles for many cellular processes in living cells. A large number of protein kinases and phosphatases have been involved in such intracellular signal transduction, as mentioned above. Herein, particular protein kinases are recognized as an important factor in cancer and many diseases due to those abnormal activities, such as hyper- or hypoactivation. If these unnatural activities of protein kinases can be used therapeutically, it could contribute to a significant development in human pathology.

For this perspective, we proposed a new concept of drug or gene delivery system responding to cellular signal (D-RECS). This system can distinguish a difference of activity in its target enzymes, including IκB kinase β, protein kinase A, Rho-associated coiled-coil kinase, and protein kinase Cα, between normal and diseased cells.[83-90] In this system, we designed an artificial gene carrier possessing a polymeric main chain and a cationic peptide side chain that is a substrate of the target enzyme. This polymer forms a polyplex with pDNA through electrostatic interactions between anionic plasmid DNA (pDNA) strand and cationic peptide substrate moieties, and the transgene transcription is totally suppressed in this polyplex. On the other hand, the polyplex is dissociated specifically in target disease cells with phosphorylation reaction on the cationic peptide side chain (**Figure 1.5**).[90] Using this concept, we successfully demonstrated D-RECS as a highly disease cell-specific gene carrier. This can be expanded to conventional disease-site targeting system for more specific and safer gene delivery system.

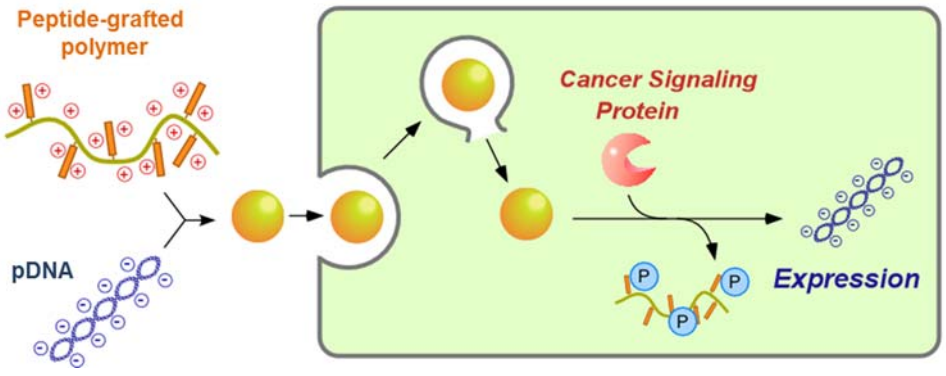


Figure 1.5. The concept of drug or gene delivery system responding to cellular signals (D-RECS).

1.4 Overview of this thesis

The focus of this thesis is on the development of more effective and specific gene delivery system responding to PKCα, based on the D-RECS concept, presenting two approaches, using cationic polymer backbone and introducing hydrophobic interaction. These approaches are able to be served as an alternative application for cancer-specific gene therapy. Moreover, I focus on PKCα as a target enzyme, which is hyperactivated in drug-resistance cancer cells, and also provide a potential as an appropriate modulator for reversal of the resistance to drug in cancer chemotherapy. The content of this thesis is organized as follows.

In chapter 2, I introduce a PKCα-responsive gene delivery system with a linear polyethylenimine (LPEI) and a PKCα-specific peptide substrate, showing quite high transgene expression responding to abnormally activated PKCα. I extent this LPEI-peptide conjugate to other various cancer cell lines, and also present the PKCα-specific gene expression *in vivo* in model mice xenografted with human lung cancer.

In chapter 3, I propose a new carrier to stabilize the polyplex through hydrophobic interaction. A simple modification of a long alkyl chain as a spacer between a LPEI main chain and a substrate peptide presents the enhanced stability of polyplex to significantly improve the PKCα-responsive transgene expression *in vitro*.

In chapter 4, I investigate the regulation of PKCα activity and their effect on reversal of the resistance to drug in drug-resistance human cancer cells. The regulation of PKCα activity exhibits increased drug accumulation, representing the potential as an alternative modulator.

In chapter 5, I summarize the contributions of this thesis

1.5 References

(1) Alberts, B.; Bray, D.; Hopkin, K.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Essential Cell Biology*, 2009.

(2) Adjei, A. A.; Hidalgo, M. *J Clin Oncol* **2005**, *23*, 5386.

(3) Ma, W. W.; Adjei, A. A. *Ca-Cancer J Clin* **2009**, *59*, 111.

(4) Kholodenko, B. N. *Nat Rev Mol Cell Bio* **2006**, *7*, 165.

(5) Bianco, R. B.; Melisi, D.; Ciardiello, F.; Tortora, G. *Eur J Cancer* **2006**, *42*, 290.

(6) Whittaker, S.; Marais, R.; Zhu, A. X. *Oncogene* **2010**, *29*, 4989.

(7) Ubersax, J. A.; Ferrell, J. E. *Nat Rev Mol Cell Bio* **2007**, *8*, 665.

(8) Blume-Jensen, P.; Hunter, T. *Nature* **2001**, *411*, 355.

(9) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. *Science* **2002**, *298*, 1912.

(10) Johnson, G. L.; Lapadat, R. *Science* **2002**, *298*, 1911.

(11) Klipp, E.; Liebermeister, W. *Bmc Neurosci* **2006**, *7*.

(12) Adams, J. A. *Chem Rev* **2001**, *101*, 2271.

(13) Cheng, H. C.; Qi, R. Z.; Paudel, H.; Zhu, H. J. *Enzyme Res* **2011**, 794089.

(14) Cohen, P. *Nat Rev Drug Discov* **2002**, *1*, 309.

(15) Fabian, M. A.; Biggs, W. H.; Treiber, D. K.; Atteridge, C. E.; Azimioara, M. D.; Benedetti, M. G.; Carter, T. A.; Ciceri, P.; Edeen, P. T.; Floyd, M.; Ford, J. M.; Galvin, M.; Gerlach, J. L.; Grotzfeld, R. M.; Herrgard, S.; Insko, D. E.; Insko, M. A.; Lai, A. G.; Lelias, J. M.; Mehta, S. A.; Milanov, Z. V.; Velasco, A. M.; Wodicka, L. M.; Patel, H. K.; Zarrinkar, P. P.; Lockhart, D. J. *Nat Biotechnol* **2005**, *23*, 329.

(16) Noble, M. E. M.; Endicott, J. A.; Johnson, L. N. *Science* **2004**, *303*, 1800.

(17) Walaas, S. I.; Greengard, P. *Pharmacol Rev* **1991**, *43*, 299.

(18) Stratakis, C. A.; Cho-Chung, Y. S. *Trends Endocrin Met* **2002**, *13*, 50.

(19) Shchemelinin, I.; Sefc, L.; Necas, E. *Folia Biol-Prague* **2006**, *52*, 81.

(20) Wu, T. T.; Hsieh, Y. H.; Wu, C. C.; Hsieh, Y. S.; Huang, C. Y.; Liu, J. Y. *Clin Chim Acta* **2007**, *382*, 54.

(21) Jornayvaz, F. R.; Shulman, G. I. *Cell Metab* **2012**, *15*, 574.

(22) Zhang, J. M.; Yang, P. L.; Gray, N. S. *Nat Rev Cancer* **2009**, *9*, 28.

(23) Kang, J. H.; Toita, R.; Kim, C. W.; Katayama, Y. *Biotechnol Adv* **2012**, *30*, 1662.

(24) Mackay, H. J.; Twelves, C. J. *Nat Rev Cancer* **2007**, *7*, 554.

(25) Newton, A. C. *J Biol Chem* **1995**, *270*, 28495.

(26) Nakashima, S. *J Biochem* **2002**, *132*, 669.

(27) Koivunen, J.; Aaltonen, V.; Peltonen, J. *Cancer Lett* **2006**, *235*, 1.

(28) Steinberg, S. F. *Physiol Rev* **2008**, *88*, 1341.

(29) Newton, A. C. *Chem Rev* **2001**, *101*, 2353.

(30) Nishizuka, Y. *Science* **1992**, *258*, 607.

(31) Berridge, M. J. *Biochim Biophys Acta* **2009**, *1793*, 933.

(32) Berridge, M. J.; Lipp, P.; Bootman, M. D. *Nat Rev Mol Cell Bio* **2000**, *1*, 11.

(33) Martiny-Baron, G.; Fabbro, D. *Pharmacol Res* **2007**, *55*, 477.

(34) Griner, E. M.; Kazanietz, M. G. *Nat Rev Cancer* **2007**, *7*, 281.

(35) Teicher, B. A. *Clin Cancer Res* **2006**, *12*, 5336.

(36) Hanauske, A. R.; Sundell, K.; Lahn, M. *Curr Pharm Design* **2004**, *10*, 1923.

(37) Aziz, M. H.; Manoharan, H. T.; Verma, A. K. *Cancer Res* **2007**, *67*, 1385.

(38) Grossoni, V. C.; Falbo, K. B.; Kazanietz, M. G.; Joffe, E. D. B. D.; Urtreger, A. J. *Mol Carcinogen* **2007**, *46*, 381.

(39) Aziz, M. H.; Manoharan, H. T.; Sand, J. M.; Verma, A. K. *Mol Carcinogen* **2007**, *46*, 646.

(40) Dennis, J. U.; Dean, N. M.; Bennett, C. F.; Griffith, J. W.; Lang, C. M.; Welch, D. R. *Cancer Lett* **1998**, *128*, 65.

(41) Mapelli, E.; Banfi, P.; Sala, S.; Sensi, M.; Supino, R.; Zunino, F.; Gambetta, R. A. *Int J Cancer* **1994**, *57*, 281.

(42) Murray, N. R.; Weems, J.; Braun, U.; Leitges, M.; Fields, A. P. *Cancer Res* **2009**, *69*, 656.

(43) Benhadji, K. A.; Serova, M.; Ghoul, A.; Cvitkovic, E.; Le Tourneau, C.; Ogbourne, S. M.; Lokiec, F.; Calvo, F.; Hammel, P.; Faivre, S.; Raymond, E. *Brit J Cancer* **2008**, *99*, 1808.

(44) Lee, K. W.; Kim, S. G.; Kim, H. P.; Kwon, E.; Yons, J.; Choi, H. J.; Park, J. H.; Kang, B. C.; Im, S. A.; Kim, T. Y.; Kim, W. H.; Bang, Y. J. *Cancer Res* **2008**, *68*, 1916.

(45) Lahn, M.; Paterson, B. M.; Sundell, K.; Ma, D. *Eur J Cancer* **2004**, *40*, 10.

(46) von Burstin, V. A.; Xiao, L. Q.; Kazanietz, M. G. *Mol Pharmacol* **2010**, *78*, 325.

(47) Bin Hafeez, B.; Zhong, W. X.; Weichert, J.; Dreckschmidt, N. E.; Jamal, M. S.; Verma, A. K. *Cancer Res* **2011**, *71*, 2318.

(48) Gavrielides, M. V.; Frijhoff, A. F.; Conti, C. J.; Kazanietz, M. G. *Curr Drug Targets* **2004**, *5*, 431.

(49) Benavides, F.; Blando, J.; Perez, C. J.; Garg, R.; Conti, C. J.; DiGiovanni, J.; Kazanietz, M. G. *Cell Cycle* **2011**, *10*, 268.

(50) Eder, A. M.; Sui, X. M.; Rosen, D. G.; Nolden, L. K.; Cheng, K. W.; Lahad, J. P.; Kango-Singh, M.; Lu, K. H.; Warneke, C. L.; Atkinson, E. N.; Bedrosian, I.; Keyomarsi, K.; Kuo, W. L.; Gray, J. W.; Yin, J. C. P.; Liu, J. S.; Halder, G.; Mills, G. B. *P Natl Acad Sci USA* **2005**, *102*, 12519.

(51) Lahn, M.; Kohler, G.; Sundell, K.; Su, C.; Li, S. Y.; Paterson, B. M.; Bumol, T. F. *Oncology-Basel* **2004**, *67*, 1.

(52) Sun, R. H.; Gao, P.; Chen, L.; Ma, D. L.; Wang, J. M.; Oppenheim, J. J.; Zhang, N. *Cancer Res* **2005**, *65*, 1433.

(53) Zhang, L.; Huang, J.; Yang, N.; Liang, S.; Barchetti, A.; Giannakakis, A.; Cadungog, M. G.; O'Brien-Jenkins, A.; Massobrio, M.; Roby, K. F.; Katsaros, D.; Gimotty, P.; Butzow, R.; Weber, B. L.; Coukos, G. *Cancer Res* **2006**, *66*, 4627.

(54) Regala, R. P.; Weems, C.; Jamieson, L.; Khoor, A.; Edell, E. S.; Lohse, C. M.; Fields, A. P. *Cancer Res* **2005**, *65*, 8905.

(55) Stallings-Mann, M.; Jamieson, L.; Regala, R. P.; Weems, C.; Murray, N. R.; Fields, A. P. *Cancer Res* **2006**, *66*, 1767.

(56) Bae, K. M.; Wang, H.; Jiang, G. H.; Chen, M. G.; Lu, L.; Xiao, L. *Cancer Res* **2007**, *67*, 6053.

(57) Paz-Ares, L.; Douillard, J. Y.; Koralewski, P.; Manegold, C.; Smit, E. F.; Reyes, J. M.; Chang, G. C.; John, W. J.; Peterson, P. M.; Obasaju, C. K.; Lahn, M.; Gandara, D. R. *J Clin Oncol* **2006**, *24*, 1428.

(58) Herbst, R. S.; Oh, Y.; Wagle, A.; Lahn, M. *Clin Cancer Res* **2007**, *13*, 4641s.

(59) Lang, W. H.; Wang, H. M.; Ding, L.; Xiao, L. *Cell Signal* **2004**, *16*, 457.

(60) Pardo, O. E.; Wellbrock, C.; Khanzada, U. K.; Aubert, M.; Arozarena, I.; Davidson, S.; Bowen, F.; Parker, P. J.; Filonenko, V. V.; Gout, I. T.; Sebire, N.; Marais, R.; Downward, J.; Seckl, M. J. *Embo J* **2006**, *25*, 3078.

(61) Dempsey, E. C.; Cool, C. D.; Littler, C. M. *Pharmacol Res* **2007**, *55*, 545.

(62) Cohen, E. E. W.; Lingen, M. W.; Zhu, B. M.; Zhu, H. Y.; Straza, M. W.; Pierce, C.; Martin, L. E.; Rosner, M. R. *Cancer Res* **2006**, *66*, 6296.

(63) Steinhart, R.; Kazimirsky, G.; Okhrimenko, H.; Ben-Hur, T.; Brodie, C. *Glia* **2007**, *55*, 224.

(64) Uht, R. M.; Amos, S.; Martin, P. M.; Riggan, A. E.; Hussaini, I. M. *Oncogene* **2007**, *26*, 2885.

(65) Martin, V.; Herrera, F.; Carrera-Gonzalez, P.; Garcia-Santos, G.; Antolin, I.; Rodriguez-Blanco, J.; Rodriguez, C. *Cancer Res* **2006**, *66*, 1081.

(66) Ogbomo, H.; Biru, T.; Michaelis, M.; Loeschmann, N.; Doerr, H. W.; Cinatl, J. *Biochem Pharmacol* **2011**, *81*, 251.

(67) Aeder, S. E.; Martin, P. M.; Soh, J. W.; Hussaini, I. M. *Oncogene* **2004**, *23*, 9062.

(68) Thuringer, D.; Hammann, A.; Benikhlef, N.; Fourmaux, E.; Bouchot, A.; Wettstein, G.; Solary, E.; Garrido, C. *J Biol Chem* **2011**, *286*, 3418.

(69) Martin, P. M.; Hussaini, I. M. *Expert Opin Ther Tar* **2005**, *9*, 299.

(70) Neri, A.; Marmiroli, S.; Tassone, P.; Lombardi, L.; Nobili, L.; Verdelli, D.; Civallero, M.; Cosenza, M.; Bertacchini, J.; Federico, M.; De Pol, A.; Deliliers, G. L.; Sacchi, S. *Leukemia Lymphoma* **2008**, *49*, 1374.

(71) Rizvi, M. A.; Ghias, K.; Davies, K. M.; Ma, C. G.; Weinberg, F.; Munshi, H. G.; Krett, N. L.; Rosen, S. T. *Mol Cancer Ther* **2006**, *5*, 1783.

(72) Alkan, S.; Huang, O.; Ergin, M.; Denning, M. F.; Nand, S.; Maududi, T.; Paner, G. P.; Ozpuyan, F.; Izban, K. F. *Am J Hematol* **2005**, *79*, 97.

(73) Barragan, M.; de Frias, M.; Iglesias-Serret, D.; Campas, C.; Castano, E.; Santidrian, A. F.; Coll-Mulet, L.; Cosialls, A. M.; Domingo, A.; Pons, G.; Gil, J. *J Leukocyte Biol* **2006**, *80*, 1473.

(74) Velcheti, V.; Tamma, S.; Larned, Z. L.; Fuloria, J.; Li, L.; Rodwig, F. R.; Cole, J. T.; Samaniego, F.; Prakash, O. *Blood* **2008**, *112*, 726.

(75) Redig, A. J.; Platanias, L. C. *Leukemia Lymphoma* **2008**, *49*, 1255.

(76) Podar, K.; Raab, M. S.; Zhang, J.; McMillin, D.; Breitkreutz, I.; Tai, Y. T.; Lin, B. K.; Munshi, N.; Hideshima, T.; Chauhan, D.; Anderson, K. C. *Blood* **2007**, *109*, 1669.

(77) Konopatskaya, O.; Poole, A. W. *Trends Pharmacol Sci* **2010**, *31*, 8.

(78) da Rocha, A. B.; Mans, D. R.; Regner, A.; Schwartsmann, G. *Oncologist* **2002**, *7*, 17.

(79) Hanauske, A. R.; Sundell, K.; Lahn, M. *Curr Pharm Des* **2004**, *10*, 1923.

(80) Chambers, T. C.; Mcavoy, E. M.; Jacobs, J. W.; Eilon, G. *J Biol Chem* **1990**, *265*, 7679.

(81) Budworth, J.; Gant, T. W.; Gescher, A. *Brit J Cancer* **1997**, *75*, 1330.

(82) Idriss, H.; Urquidi, V.; Basavappa, S. *Cancer Chemoth Pharm* **2000**, *46*, 287.

(83) Katayama, Y.; Fujii, K.; Ito, E.; Sakakihara, S.; Sonoda, T.; Murata, M.; Maeda, M. *Biomacromolecules* **2002**, *3*, 905.

(84) Oishi, J.; Kawamura, K.; Kang, J. H.; Kodama, K.; Sonoda, T.; Murata, M.; Niidome, T.; Katayama, Y. *J Control Release* **2006**, *110*, 431.

(85) Kawamura, K.; Oishi, J.; Kang, J. H.; Kodama, K.; Sonoda, T.; Murata, M.; Niidome, T.; Katayama, Y. *Biomacromolecules* **2005**, *6*, 908.

(86) Kang, J. H.; Asai, D.; Kim, J. H.; Mori, T.; Toita, R.; Tomiyama, T.; Asami, Y.; Oishi, J.; Sato, Y. T.; Niidome, T.; Jun, B.; Nakashima, H.; Katayama, Y. *J Am Chem Soc* **2008**, *130*, 14906.

(87) Asai, D.; Tsuchiya, A.; Kang, J. H.; Kawamura, K.; Oishi, J.; Mori, T.; Niidome, T.; Shoji, Y.; Nakashima, H.; Katayama, Y. *J Gene Med* **2009**, *11*, 624.

(88) Toita, R.; Kang, J. H.; Kim, J. H.; Tomiyama, T.; Mori, T.; Niidome, T.; Jun, B.; Katayama, Y. *J Control Release* **2009**, *139*, 133.

(89) Tomiyama, T.; Toita, R.; Kang, J. H.; Asai, D.; Shiosaki, S.; Mori, T.; Niidome, T.; Katayama, Y. *J Control Release* **2010**, *148*, 101.

(90) Toita, R.; Kang, J. H.; Tomiyama, T.; Kim, C. W.; Shiosaki, S.; Niidome, T.; Mori, T.; Katayama, Y. *J Am Chem Soc* **2012**, *134*, 15410.

CHAPTER 2

Smart gene carrier: its efficient transgene regulation responding to PKCα *in vitro* and *in vivo*

2.1 Introduction

Gene therapy has shown great promise over the past decades for preventing and treating genetic disorders such as hereditary diseases and cancers. The first FDA approved clinical trial was performed in 1990, treating a severe immune system deficiency. Since then, in 2013, over 1,700 clinical trials have been conducted by diverse approaches for gene therapy.[1] The most compelling aspect for gene therapy comes from replacing and correcting a functional gene with a deleterious mutant allele as a specific treatment, not just to deal with a symptomatic treatment of diseases. However, gene therapy has encountered many issues in laboratory studies and clinical trials concerning the safety and effectiveness, such as its undesirable immune responses and the low efficacy of gene transfection. Although the limitations of gene therapy still exist, many researchers have been devoted to the discovery and development of strategies to overcome these limitations. Indeed, the recent successes, in 2006-2013, have led to a renewed interest in this field.[2-5]

Generally, gene delivery systems have investigated under major two approaches, using viral- and non-viral vectors. Although viral vectors, including retrovirus, adenovirus, adeno-associated virus, and herpes simplex virus, have led the majority of clinical trials,[1] it has shown severe intrinsic obstacles, such as dangerous immune reactions and limitations in target-cell specificity.[6-11] In contrast, non-viral vectors, including cationic lipids, polymers, dendrimers, and peptides, present attractive alternatives with those desirable properties, such as safety and simplicity of preparation.[12-15] Therefore, significant strategies have been demonstrated as non-viral delivery system. Especially, one prominent challenge is that polymeric vectors, including poly(L-lysine) (PLL),[16-19] polyethylenimine (PEI) (e.g. linear PEI, branched

PEI),[20-24] polymethacrylate,[25-29] carbohydrate-based polymers (e.g. chitosan, dextran),[30-34] linear poly(amido-amine) (PAA),[35-37] and biodegradable polymers,[38-42] are intensively studied for gene delivery system.[7,13]

Another important aspect for gene delivery system is target cell- and tissue-specific delivery for efficient expression of transgene. Various approaches have merged to develop a proper gene delivery system, in which transgene can be specifically expressed at pathological sites. These approaches lead to especially an increasing application to cancer therapy, consisting of following two methods: "passive targeting" and "active targeting" (**Figure 2.1**).[14,43]

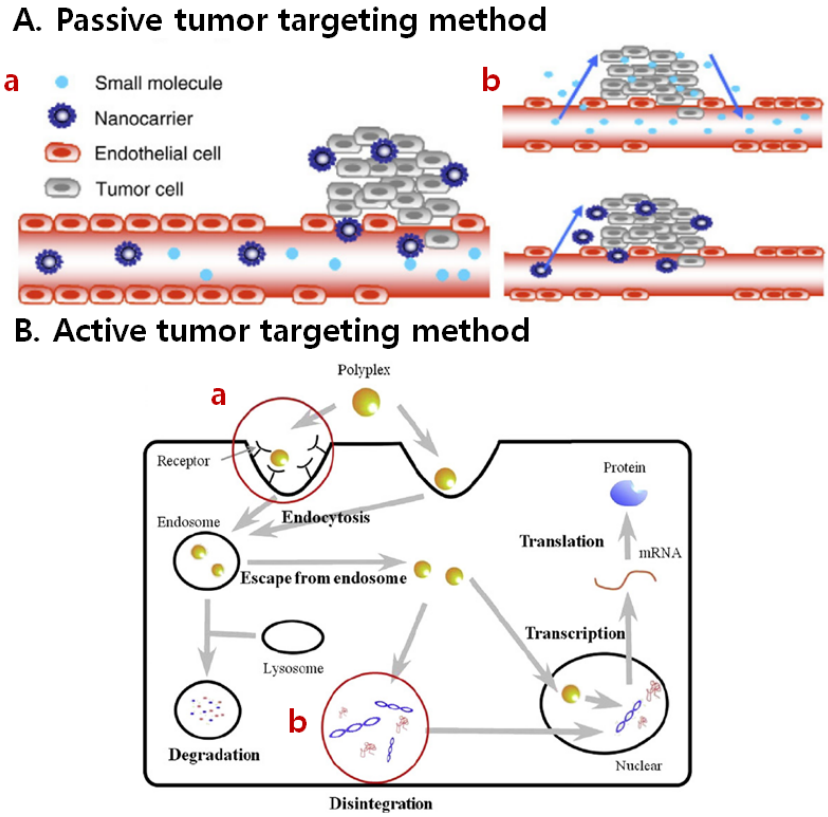


Figure 2.1. Scheme of the passive tumor targeting method (A) and of the active tumor targeting method (B). (A) Nanocarrier can pass through leaky, tortuous, and heterogeneous tumor blood vessels and tend to accumulate in tumors, but none or very few pass through normal blood vessels showing a well-organized and functional structure (enhanced permeability and retention (EPR) effect). (B) In tumor cells, hyperactivated cell surface receptors or intracellular signals are identified and they become good targets for tumor-targeted gene delivery. (a) For targeting hyperactivated receptors, carriers containing a ligand specific to the receptor are used. After the binding of carrier to each ligand-specific receptor, the carrier and receptor are transferred into the cytosol by endocytosis. (b) Phosphorylation or cleavage of nanoparticles by hyperactivated target enzymes leads to their disintegration.

Although these various approaches and efforts have been directed to the development of a proper gene delivery system, there still remain significant obstacles to realizing an effective delivery of therapeutic genes to target sites.[44-46] For overcoming this hindrance, we have developed a novel concept of gene carrier that can respond to malfunctioning intracellular signaling in diseased cells, called D-RECS as mentioned in **Chapter 1**. These gene carriers are composed of a hydrophilic polymeric main chain grafted with a cationic peptide which is a substrate of protein kinases activated specifically in the diseased cells. These carriers form the polyplexes with anionic plasmid (pDNA) through electrostatic interaction between the anionic DNA strand and the cationic peptide, and the gene expression is effectively suppressed in the polyplexes formation. Once the polyplexes are introduced into diseased cells, phosphorylation of the peptide substrate is conducted by protein kinases specifically activated in diseased cells and then leads to dissociation of the polyplexes for gene expression due to the decrease of the cationic charge of the grafted peptide (**Figure 2.1A**). In efforts toward achieving this intracellular signal-responsive system, we have previously reported several gene carriers that respond to target protein kinases, such as IκB kinase β, protein kinase A, Rho-associated coiled-coil kinase, and protein kinase Cα (PKCα).[47-53]

Among several target kinases, PKCα is one of the most prominent targets for the treatment of cancer, which is hyperactivated in various cancer cells as mentioned in **Chapter 1**. Consequently, we have introduced a PKCα-responsive peptide (Alphatomega; FKKQG**S**FAKKK-NH_2), and this peptide substrate revealed the highly specificity and efficiency to PKCα, comparing relatively low phosphorylation efficiency by other PKC isozymes (**Table 2.1**).[54] This contributes to broad applications for the diagnosis of tumors and the anticancer therapies.

Table 2.1. Kinetic parameters (K_m, V_{max}, and V_{max}/K_m) for Alphatomega.[54]

Isozymes	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
α	17.4	138	7.9
βI	35.6	175	4.9
βII	59.9	38.6	0.6
γ	34.7	47.0	1.4
δ	42.2	8.8	0.2
ε	22.9	66.2	2.9
η	16.6	41.8	2.5
θ	34.0	7.6	0.2
ζ	20.5	17.4	0.9
ι/λ	25.8	90.4	3.5

We have recently introduced a PKCα-responsive gene carrier in the form of a linear polyethylenimine (LPEI)–peptide conjugate. Here, a PKCα-specific peptide substrate (Alphatomega) is modified with LPEI, which is one of the most widely used cationic polymer for gene delivery due to its high transfection efficacy via endosomal escape (**Figure 2.2B**).[55-60] I present detailed characterization of its specific transgene expression responding to target PKCα in both *in vitro* and *in vivo*. I examined the efficacy of transgene regulation responding to intracellular PKCα in skin, lung, liver, and breast cancer cell lines, and also exhibited PKCα-specific gene expression in human lung cancer *in vivo*. In addition, I observed intracellular trafficking of polyplexes, showing the correlation between those endosomal escape ability and transfection efficacy.

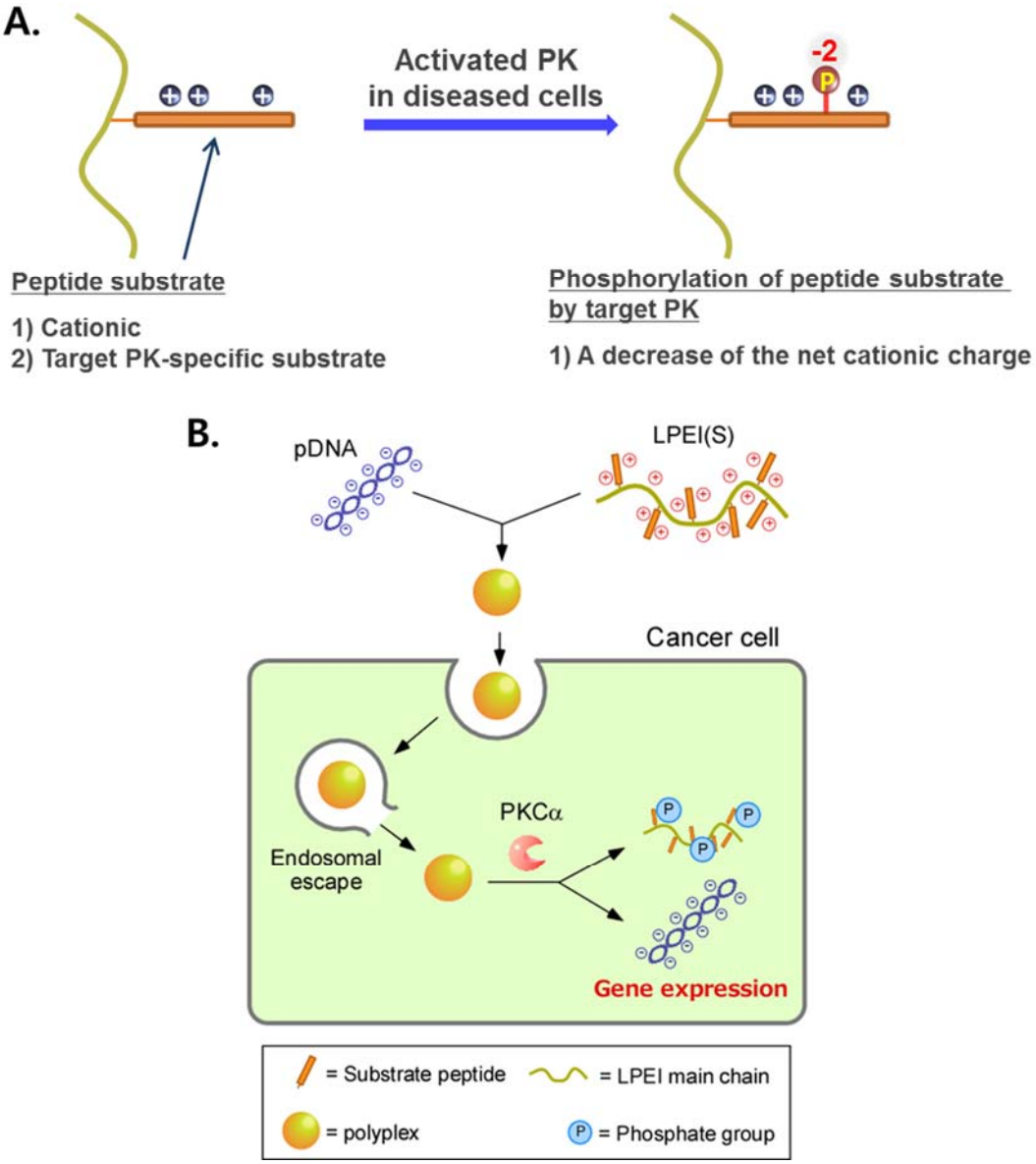
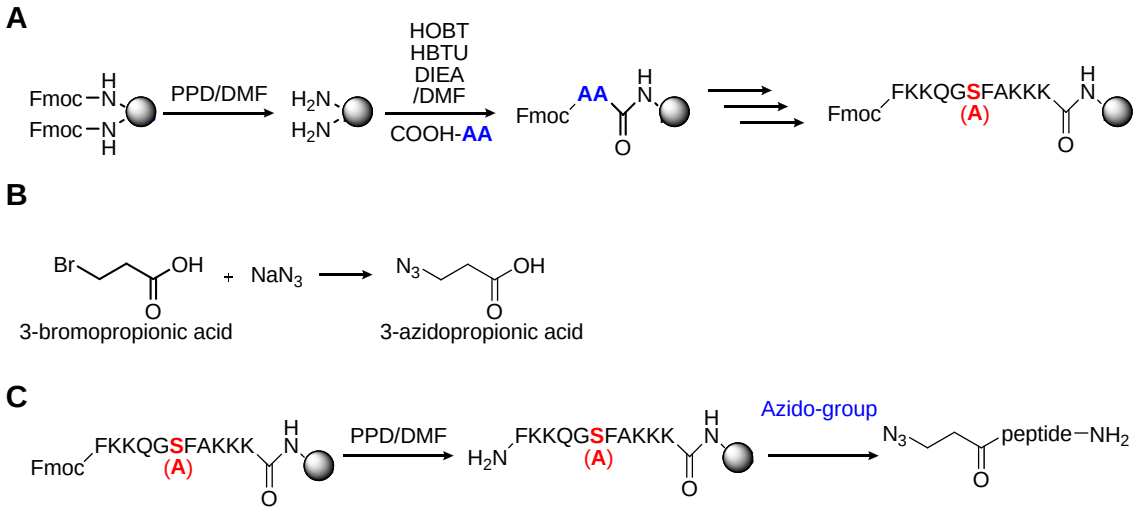


Figure 2.2. Scheme of the cancer cell-targeted gene delivery system responding to intracellular PKCα which is hyperactivated in various cancer cells and tissues. (A) The phosphorylation of the peptide substrate induces a decrease of the net charge of carrier, (B) The LPEI-peptide conjugate (LPEI-C_2(S)) comprises a LPEI main chain and a PKCα-specific peptide substrate side chain. Transcription of the pDNA is suppressed by the formation of a polyplex. After the phosphorylation reaction with PKCα, however, the pDNAs are released from the polyplex because of the decrease in net cationic charge, leading to transgene expression.[59]

2.2 Results and discussion

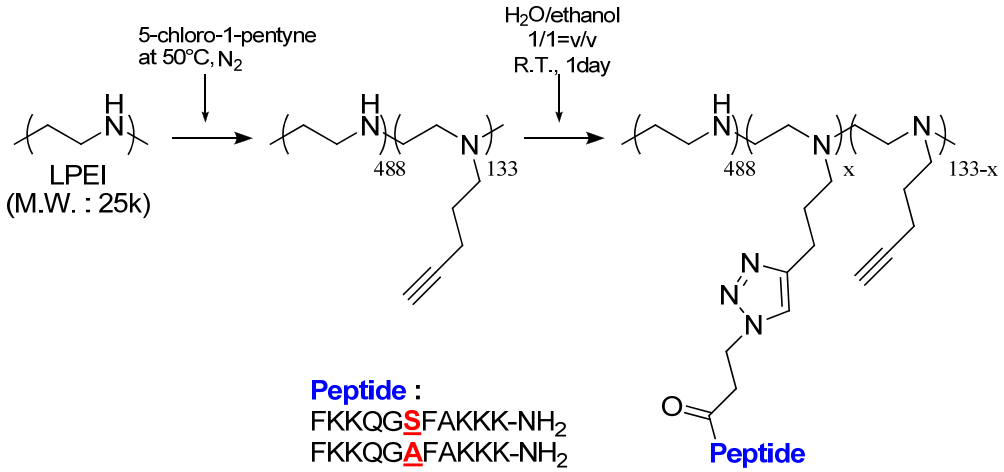
2.2.1 Synthesis of PKCα-responsive polymer

A peptide substrate (Alphatomega; FKKQG**S**FAKKK-NH_2), which is specifically recognized by intracellular PKCα, was synthesized using an automatic peptide synthesizer according to standard Fmoc-chemistry procedures. The Azido-group modified peptide substrate was then obtained as follows: (1) 3-bromopropionic acid was substituted with azide-group by the addition of sodium azide, (2) the following *N*-terminus azido peptide was synthesized with crude 3-azidopropionic acid on the *N*-terminus of the peptide substrate as shown in **Scheme 2.1**.[47,59] As a negative control, serine residue on the phosphorylation site of the peptide substrate was substituted for alanine (FKKQG**A**FAKKK-NH_2) following the same procedures.



Scheme 2.1. Synthetic scheme of the azido peptide substrate. Synthesis of peptide substrate (Alphatomega) (A), 3-azidopropionic acid (B), and the *N*-terminus azido peptide substrate, which is specifically responsive to PKCα (C).
* ● : polystyrene, AA: amino acid

The modification of peptide substrates on LPEI was performed via a two-step synthetic procedure as shown in **Scheme 2.2**.[59] The contents of the peptide substrate in LPEI-C_2(S) and LPEI-C_2(A) were calculated by TNBS assay, 6.16 % and 6.13 %, respectively (**Table 2.1**).



Scheme 2.2. Synthetic scheme for LPEI-peptide conjugates.[a]

[a]Reagents and conditions: (i) 5-chloro-1-pentyne, DBU, and dry DMSO at 50°C; (ii) *N*-terminus azido-peptide, copper(II) pentahydrate, sodium ascorbate, and H_2O/ethanol = 1/1 at room temperature.

Table 2.1. Molecular parameters of LPEI-peptide conjugate.

samples	peptide sequence	peptide content [a] / mol %	peptide no. / chain	M_w [b] / 10^4 g/mol
LPEI-C_2(S)	-FKKQG**S**FAKKK-NH_2	6.16	35	8.2
LPEI-C_2(A)	-FKKQG**A**FAKKK-NH_2	6.13	35	8.2

[a] Determined by TNBS assay. [b] Calculated from peptide and pentyne contents and M_w of the parent LPEI.

2.2.2 PKCα-specific reactivity of LPEI-peptide conjugate

Abnormal activities of PKCα have been implicated in cancer formation and progression as mentioned in **Chapter 1**. To verify the activities of PKCα in cancer cells, I examined the phosphorylation level of the PKCα-specific peptide substrate with cancer cell lysates at a 200 μg/mL protein concentration. Cell lysates were prepared from various cancer cells, including mouse skin, human lung, liver, brain, and breast cancers, and the phosphorylation of the peptide substrate was evaluated by MALDI-TOF-MS analysis. As shown in **Figure 2.3**, the distinct phosphorylation of the peptide substrate indicated relatively higher level (48% to 88%) than that in normal tissue lysates as reported previously.[51,54] These specific phosphorylation reaction, possessing three- to eight-fold higher activities than normal, may provide the rationale for targeting hyperactivated PKCα in cancers.

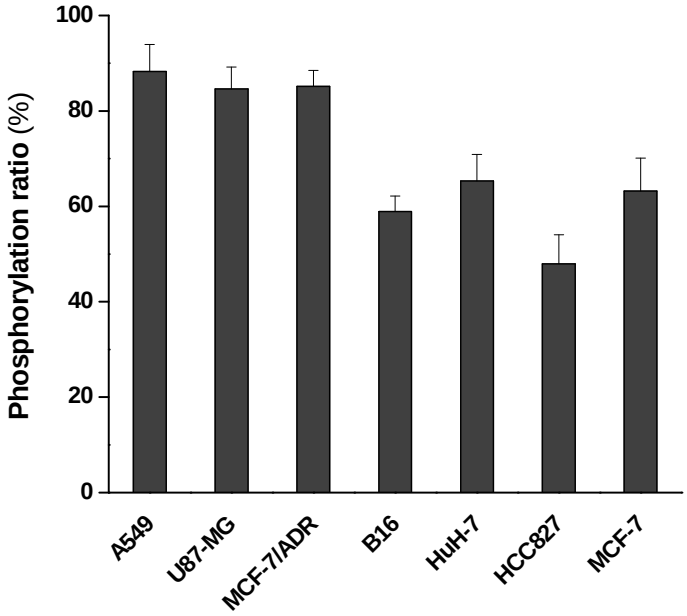


Figure 2.3. Phosphorylation ratio of the peptide substrate in various cancer cell lysates. The sample was identified by an increase in the m/z-value of +80 Da.

I investigated whether the reactivity was affected by the modification of the peptide substrate onto the LPEI main chain. The phosphorylation of the peptide substrate was identified by a coupled enzyme assay, which detects the decrease of

absorbance at 340 nm resulting from NADH oxidation caused by the consumption of ATP during the phosphorylation reaction of the peptide substrate.[52,61] The oxidation of NADH caused by the phosphorylation of the peptide substrate in the presence of PKCα was observed in both Alphatomega and LPEI-C_2(S). Although the decrease rate of absorbance at 340 nm in LPEI-C_2(S) was somewhat slower than that in Alphatomega, LPEI-C_2(A) didn't show any change of the absorbance as show in **Figure 2.4**.[59] This result demonstrated convincingly that the grafted peptide was specifically phosphorylated by target PKCα after modification onto LPEI main chain.

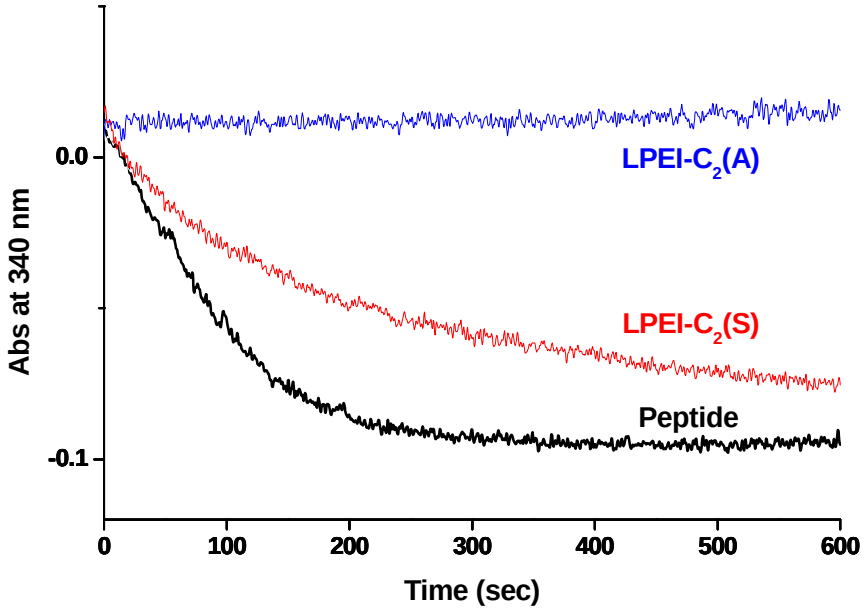


Figure 2.4. Reactivity of LPEI-peptide conjugates and the peptide substrate (H-FKKQG**S**FAKKK-NH_2) toward PKCα monitored by a coupled enzyme assay. The phosphorylation reaction was identified from the decrease of absorbance at 340nm by NADH oxidation.

2.2.3 Efficient transgene expression responding to PKCα *in vitro*

Prior to verifying *in vitro* transgene expression, the cytotoxicity of polyplexes was evaluated using a cell counting kit (WST-8) in B16 cells. As shown in **Figure 2.5**, the cytotoxicity was not observed in those of polyplexes with both LPEI-C_2(S) and LPEI-C_2(A), over a broad range of the *N/P* ratios.

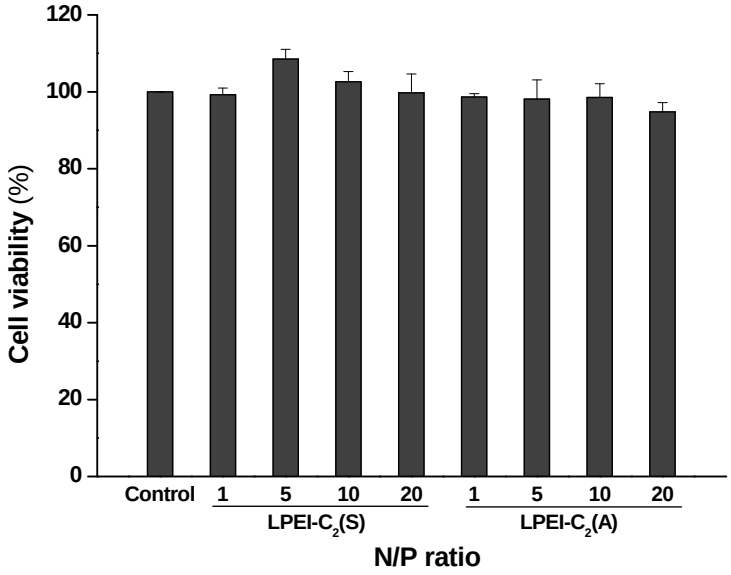


Figure 2.5. Cell viability assay for the evaluation of cytotoxicity of LPEI-peptide conjugates in B16 cells.

In order to confirm the effective transgene regulation of the LPEI-peptide conjugate *in vitro*, transfection efficacy was then evaluated by using luciferase encoding pDNA in various cancer cells. Polyplexes was prepared at the *N/P* ratios of 7 and 10, which are optimal ratios in previous investigation, with pDNA. LPEI and LPEI-C_2(A) polyplexes were used as a positive and negative control, respectively. As shown in **Figure 2.6A**, transgene expression in LPEI-C_2(S) polyplexes showed clearly higher than LPEI-C_2(A) at both of the *N/P* ratios of 7 and 10 in human U87-MG glioma cells possessing the relatively high PKCα activity. Significantly, LPEI-C_2(S) polyplexes showed a 100 time higher transgene expression than LPEI- C_2(A) at the *N/P* ratio of 10.

In addition, PKCα-specific transgene expression of polyplexes was examined in other cancer cells, in which the ratio of transgene expression level between both polyplexes [LPEI-C_2(S)/LPEI-C_2(A)] was described in terms of signal responsiveness. As shown in **Figure 2.6B**, the transgene expression of LPEI-C_2(S) polyplexes were much higher than those of LPEI-C_2(A) polyplexes, depending on the basal PKCα activities in each cancer cell, and the enhancement of the expression level with PKCα phosphorylation was more than 10 to 100 times higher compared with a negative control. Based on these results, transgene expression of polyplexes was effectively regulated by target PKCα, showing the significantly enhanced efficacy than our previous PPC carrier which includes polyacrylamide backbone (ca. 10 times).[51,52,61]

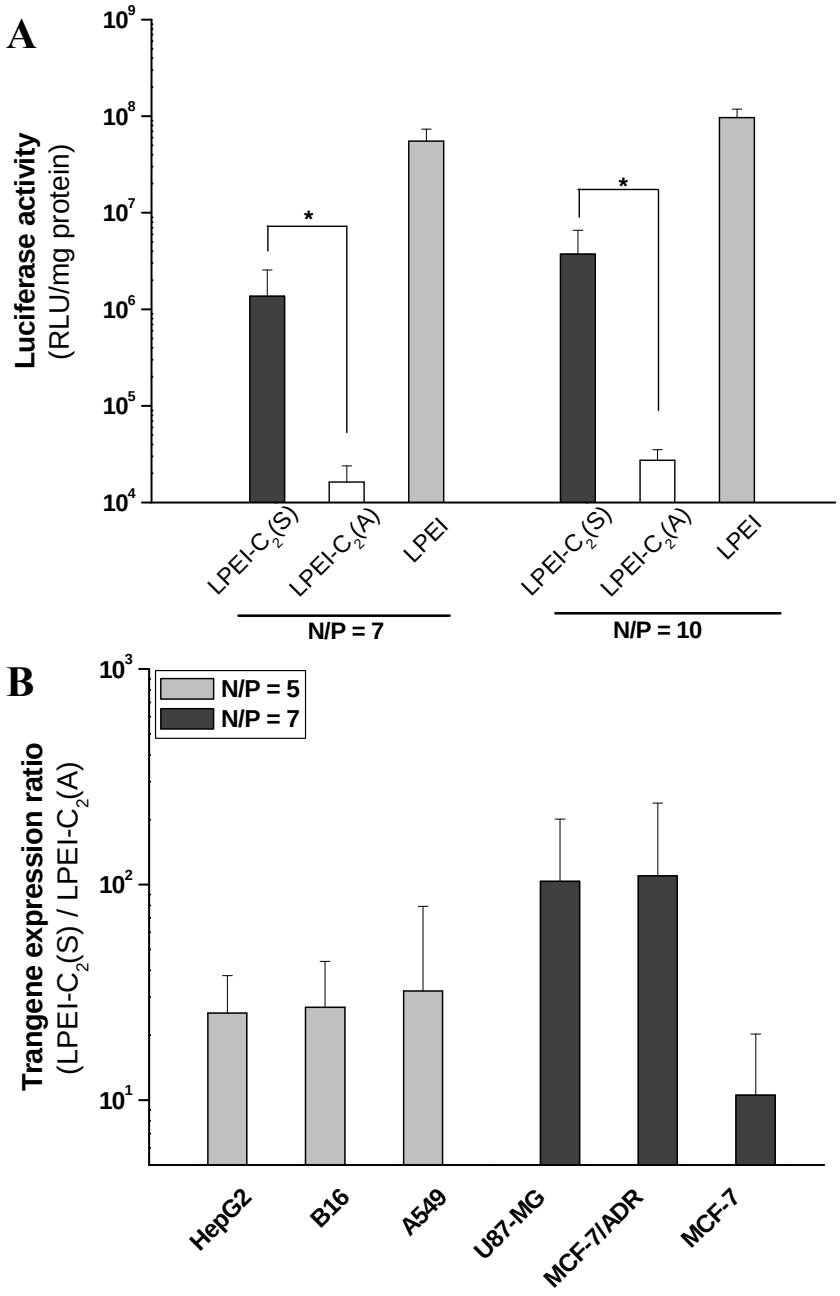


Figure 2.6. Transfection of polyplexes with luciferase encoding pDNA into various cell lines. (A) Transfection of LPEI-C_2(S) and LPEI–C_2(A) polyplexes at the *N/P* ratios of 7 and 10 in U87-MG cells. (B) Relative transgene expression ratio between LPEI-C_2(S) to LPEI–C_2(A).

2.2.4 Intracellular trafficking of polyplexes

To investigate the origin of high signal responsiveness in LPEI-C_2(S), intracellular trafficking of the polyplexes was carried out. The cellular localization of polyplexes was monitored by confocal microscopy in A549 cells. pDNA was labeled with Cy5, nuclei and late endosome/lysosomes were stained with Hoechst 33342 and LysoTracker Green, respectively.

The uptake and subcellular distribution of polyplexes was shown in **Figure 2.7**. Here, LPEI and LPEI-pentyne polyplexes were used as a positive and negative control, respectively. The results indicated that LPEI polyplexes showed effective cellular uptake via endocytosis and endosomal escape with a strong pH buffering capacity of PEI units at around 5-6 in endosome causing the so-called proton sponge effect,[62-64] whereas LPEI-pentyne polyplexes were not observed the significant subcellular localization due to the weak complexation with pDNA as described previously.[59] Although LPEI-C_2(S) polyplexes localized somewhat in late endosome/lysosomes, the most efficient cellular uptake was observed and some of them showed clear endosomal escape. The escaped polyplexes from endosomal/lysosomal compartments may lead to the efficient regulation of transgene expression in the LPEI-C_2(S), showing the significant PKCα-specific expression as shown in **Figure 2.6**. In addition, the relatively low endosomal escaping of LPEI-C_2(S) than LPEI polyplexes agreed well with the difference of transgene expression levels between LPEI-C_2(S) and LPEI polyplexes as shown in **Figure 2.6**.

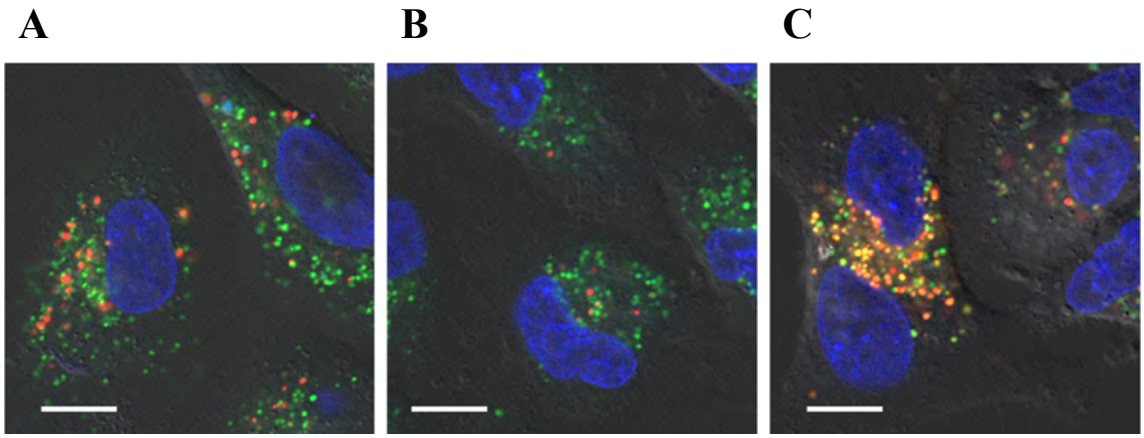


Figure 2.7. Intracellular distribution of (A) LPEI, (B) LPEI-pentyne, and (C) LPEI-C_2(S) polyplexes in A549 cells. Confocal images were taken at 18 h after 6 h transfection of polyplexes, respectively. pDNA was labeled with Cy5 (red). Late endosomes/lysosomes and the nuclei were stained with LysoTracker Green (green) and Hoechst 33342 (blue), respectively. The scale bar represents 20 μm.

To assess the extent of endosomal escape of LPEI-C_2(S) polyplexes, I then investigated that the time-dependent intracellular distribution of LPEI-C_2(S) polyplexes in A549 cells. As shown in **Figure 2.8**, after 1 h incubation, the red fluorescent dots resulting from pDNA were initially observed on the interior and/or exterior of the cell membrane. The increased red fluorescent dots were then observed in the cytoplasm over a period of 24 h. In addition, after 24 h incubation, a large number of red fluorescent dots were observed as shown in **Figure 2.8B**. This indicated that this LPEI-C_2(S) polyplexes were well internalized via endocytosis and continued to accumulation in the cytoplasm. Although endosomal escape of the polyplexes was not complete and still appeared in late endosome/lysosomes, which represented the yellow fluorescent dot, the efficacy of endosomal escape was consistent with other researchers' reports.[65-69]

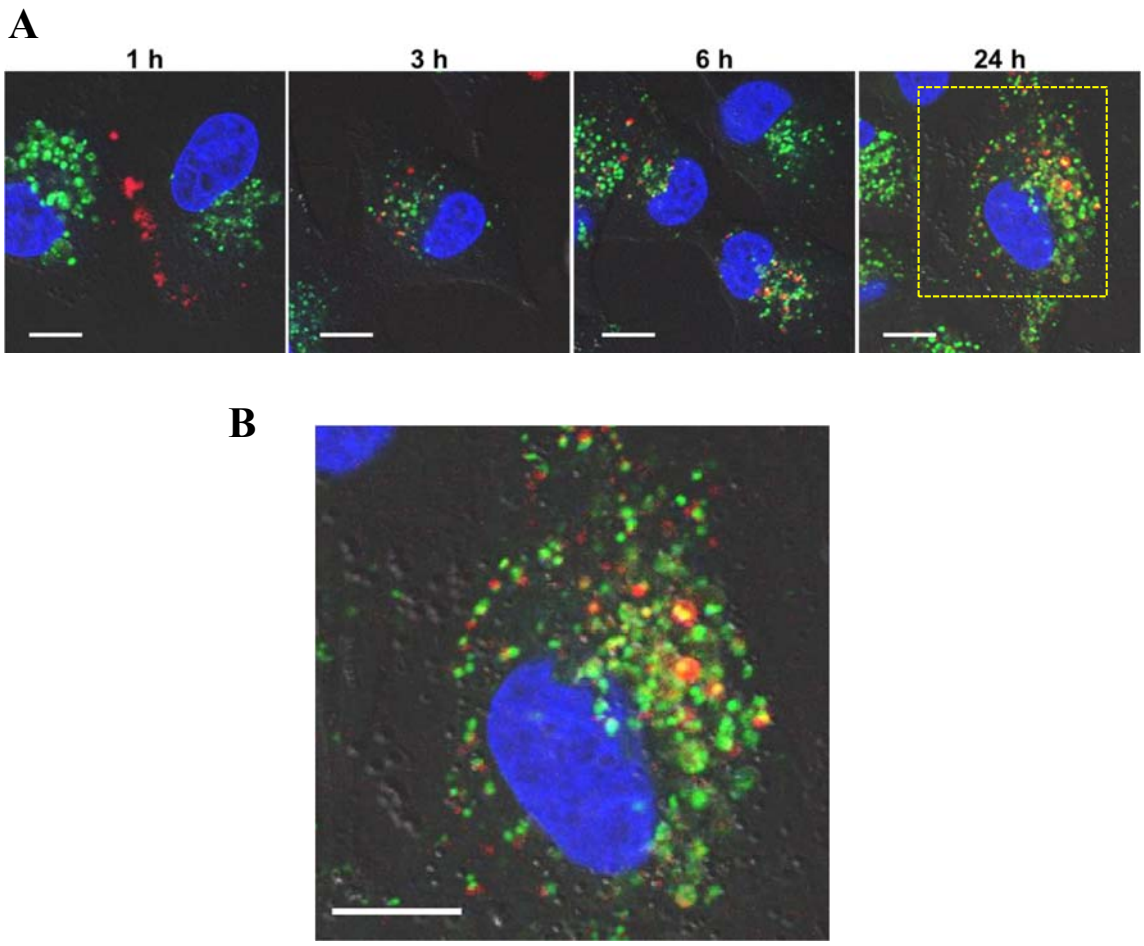


Figure 2.8. Time-course distribution of LPEI-C_2(S) polyplexes in A549 cells. (A) Confocal images were taken at 1, 3, 6, and 24 h after 6 h transfection of polyplexes. (B) Higher magnification of the yellow boxed areas in A. The scale bar represents 20 μm.

2.2.5 Cancer-specific transgene expression *in vivo*

I investigated whether the LPEI-peptide conjugate can mediate the transgene expression *in vivo* by using model mice xenografted with human A549 lung adenocarcinoma, which showed the relatively high PKCα activity. The polyplexes forming with luciferase-encoding pDNA were directly injected into the tumor tissue or normal subcutaneous tissue. The results were shown in **Figure 2.9**. As expected, the significant transgene expression was observed in LPEI-C_2(S) polyplexes (5/5), whereas no expression was observed in LPEI-C_2(A) (0/4). Furthermore, the expression of both

polyplexes was completely suppressed in the normal subcutaneous tissue. These results were completely consistent with our previous result,[59] and revealed that transgene expression of LPEI-C_2(S) polyplexes was effectively regulated by the hyperactivated PKCα in the tumor tissues, suggesting a clear suppression of transgene expression in the normal tissue.

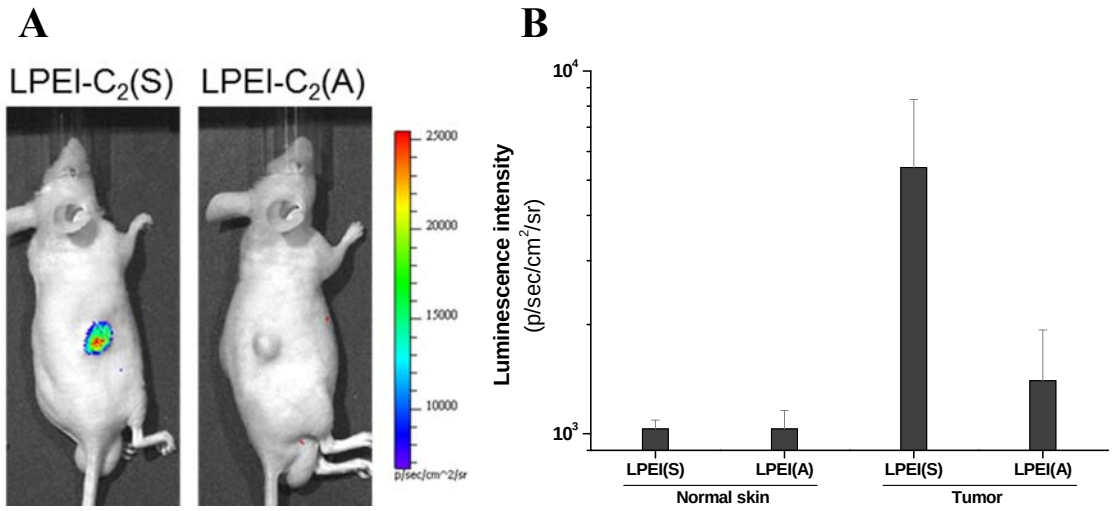


Figure 2.9. Transgene expression in A549 tumor or normal tissues. Luciferase expression was monitored by IVIS imaging system after 24 h direct injection of polyplexes. Numbers of luciferase expressing mice per total mice are as follows: 5/5 [LPEI-C_2(S)], 0/4 [LPEI-C_2(A)] in the tumor tissue, and 0/3 [both of LPEI-C2(S) and LPEI-C2(A)]

2.3 Summary

I investigated that the LPEI-peptide conjugate, consisting of the LPEI main chain and PKCα-specific peptide substrate side chain (Alphatomega). This LPEI-peptide conjugate showed the efficient transgene expression in various cancer cell lines which possessed the relatively higher activities of PKCα than normal. The efficacy of transfection expression of polyplexes in these cancer cells showed more than 10 to 100 times higher compared to a negative control. Additionally, the detailed intracellular trafficking of polyplexes revealed the efficient cellular uptake and capable of endosomal escape caused by a high buffering capacity of LPEI main chain, suggesting that their efficient transgene expression responding to PKCα in both *in vitro* and *in vivo*. Thus, this system may emerge as an alternative application for cancer-specific gene delivery.

2.4 Experimental section

Materials

Rink Amide AM resin LL (100-200 mesh, amine density of 0.48 mmol/g) and all Fmoc-protected amino acids were obtained from Novabiochem, Merck (Tokyo, Japan). 1-hydroxybenzotriazole hydrate (HOBt•H2O), O-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine (DIPEA), dichloromethane (DCM), 1-methyl-2-pyrrolidone (NMP), and piperidine were purchased from Watanabe Chemical (Hiroshima, Japan). *N*,*N*-dimethylformamide (DMF) was purchased from Kanto Chemical (Tokyo, Japan). Sodium ascorbate, copper (II) sulfate pentahydrate, sodium azide, pyridine, dimethyl sulfoxide (DMSO), and ethanol were purchased from Wako Pure Chemicals (Osaka, Japan). 5-Chloro-1-pentyne and 1,8-Diazabicyclo[5,4,0]-7-undecene (DBU) were purchased from Tokyo Kasei Industry (Tokyo, Japan). Linear polyethylenimine (LPEI; Mw = 25,000) was purchased from Polysciences, Inc. (Warrington, PA, USA). All reagents were used without further purification.

Synthesis of the peptide substrate

The peptide substrate, FKKQG**S**FAKK, was prepared by standard Fmoc-chemistry using the Rink Amide AM resin LL (100-200 mesh, amine density of 0.48 mmol/g), DIPEA as a base, HOBt/HBTU as coupling reagents, and a 20% solution of piperidine in DMF for deprotection of Fmoc group as described previously.[47,59] To apply the azido group on peptide substrate, 3-azidopropionic acid was synthesized with 3-bromoundecanoic acid by addition of 2 equivalent of sodium azide for 1 day at RT in 1:1 DMF/DMSO solution. 3-azidopropionic acid was then reacted with the *N*-terminus of the peptide in the presence of coupling reagents. The obtained peptide was purified by reverse-phase liquid chromatography and then identified by MALDI-TOF-MS. A negative control peptide, FKKQG**A**FAKK, was synthesized and purified in the same manner.

Synthesis of PKCα-responsive polymer

LPEI-peptide conjugate, LPEI-C_2(S), was synthesized with LPEI and the obtained peptide substrate by a two-step synthetic procedure as described previously.[59] LPEI was dissolved in dry DMSO (30 mL) followed by addition of pyridine, 1,8-diazabicyclo[5,4,0]-7-undecene (DBU), and 5-chloro-1-pentyne. After the reaction, DMSO was partially evaporated under reduced pressure. The residue was diluted in methanol and dialyzed by using dialysis membrane bag (MW cut off, 10,000) against methanol, then 0.05 N HCl, and finally water. The obtained polymer (LPEI-pentyne) was mixed with the azido peptide substrate, copper (II) sulfate pentahydrate, and sodium ascorbate in 800 μL of water/ethanol (1/1 = v/v) and the mixture was maintained at room temperature with continuous stirring for one day. After the reaction, the crude product was dialyzed against 0.05 N HCl and then water by using dialysis membrane bag (MW cut off, 10,000), followed by freeze-drying to obtain the conjugate. The contents of the peptide substrate were determined by trinitrobenzenesulfonic acid (TNBS) assay.[59,70] LPEI-C_2(A) was synthesized following the same manner as a negative control.

Phosphorylation assay of polymer using a coupled enzyme assay

Phosphorylation profiles of LPEI-C_2(S) and LPEI-C_2(A) were examined using a coupled enzyme assay.[52,61] The reaction was performed in 20 mM HEPES buffer [200 μM ATP, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.0 μg/mL DAG, 2.5 μg/mL PS, 0.3 mM nicotinamide adenine dinucleotide (NADH), 1 mM phosphoenolepyruvate, 10 U/μL LDH, and 4 U/μL pyruvate kinase] containing 30 μM polymer at 25 °C. Monitoring of NADH consumption was initiated by adding 1.1 ng/μL PKCα and detected with a UV/Vis spectrophotometer (UV-2550; Shimadzu, Tokyo, Japan) equipped with an SPR-8 temperature controller (Shimadzu) at 340 nm.

Plasmid DNA

A plasmid DNA, pCMV-luc2, containing a firefly luciferase cDNA fragment driven by a CMV promoter was prepared as follows. A firefly luciferase cDNA fragment was obtained from the pGL4.10[luc2] vector (Promega, WI, USA) by *Hind*III and *Xba*I. This fragment was inserted into the pcDNA3 vector (Invitrogen, CA, USA). The resulting pDNA was amplified in the *Escherichia coli* strain DH5a, isolated, and then purified using a Qiagen Plasmid Mega Kit (Qiagen, CA, USA).

Cell culture

B16 (melanoma, skin, mouse), A549 (adenocarcinoma, lung, human), HepG2 (hepatocellular carcinoma, liver, human), and HuH-7 (hepatocellular carcinoma, liver, human) cells were cultured in Dulbecco's modified Eagle's medium (DMEM). HCC827 (adenocarcinoma, lung, human) and U87-MG (Glioblastoma, brain, human) cells were cultured in RPMI 1640 medium and Eagle's minimal essential medium (MEM), respectively. MCF-7 (adenocarcinoma, breast, human) cells were cultured in MEM containing 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 μg/mL insulin. All medium contained 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Gibco Invitrogen Co., Grand Island, NY, USA). The cell was harvested in a humidified atmosphere containing 5 % CO_2 and 95 % air at 37 °C.

Cellular cytotoxicity of polyplexes

The cytotoxicity of polyplexes was assessed using a cell counting kit containing 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-8) (Dojindo Laboratories, Kumamoto, Japan) in B16 cells. Briefly, 2×10^3 cells were seeded into a 96-well plate and cultured for 24 h at 37 °C in a 5 % CO_2 incubator. The medium in each well was replaced with 100 μL of fresh medium containing polyplexes at various *N/P* ratios (0 to 20) with pDNA (0.5 μg) and different concentrations of polymer (7.1 to 52 μg/mL). After incubation for 24 h, the medium was replaced with 100 μL of fresh medium and then 10 μL of WST-8 was

added to the each well. The cells were incubated for a further 2 h. The cell viability (%) was calculated by the absorbance of the treated cells to that of the untreated control cells at 440 nm. This assay was performed in triplicate.

In vitro phosphorylation of the peptide substrate

Cells were cultured in 100-mm cell culture dishes, and scrapped in 1.5 mL buffer [10 mM HEPES, 10 % sucrose, Complete™ protease inhibitor cocktail (EDTA-Free) (Roche, Basel, Switzerland)] at 70 - 80 % confluency. The samples were centrifuged at 5000 × g at 4 °C for 10 min and the supernatant was removed. A 0.2 mL of buffer was added in the precipitate and then sonicated twice at cooled condition for 15 sec. The samples were centrifuged again at 5000 × g at 4 °C for 15 min, and the resulting supernatant was used for the phosphorylation of the peptide substrate. The phosphorylation reaction of the peptide substrate was carried out in 100 μL buffer [10 mM HEPES, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.0 μg/mL diacylglycerol (DAG), 2.5 μg/mL phosphatidylserine (PS), and 200 μM ATP] containing 30 μM peptide substrate and cell lysates at a 200 μg/mL protein concentration. The phosphorylation was allowed to proceed for 1 h at 37 °C and then the resulting solutions were analyzed by MALDI-TOF-MS.[54,71]

In vitro transfection of polyplexes

Cells were plated at a density of 2.5 - 3 × 10^4 cells in 48-well plates at 37 °C in medium containing 10 % FBS for 24 h. Polyplexes at various *N/P* ratios were prepared with pDNA (1 μg) and different concentrations of polymer for 20 min at room temperature. The medium in each well was replaced with Opti-MEM containing pDNA/polymer polyplexes at various *N/P* ratios. After incubation for 4 h, the medium was changed to medium containing 10 % FBS, and the cells were further incubated for 20 h. The cultured cells were then scraped and lysed in 100 μL of lysis buffer (20 mM Tris-HCl, pH 7.4, 0.05 % Triton-X 100, and 2 mM EDTA). A 10-μL aliquot of the lysate solution was used for measuring chemiluminescence in a MiniLumat LB 9506 (EG & G Berthold, Wildbad, Germany) directly after mixing with 40 μL of the luciferin substrate. The results are presented as relative luminescence units (RLU)/mg total protein.

Confocal laser scanning microscope (CLSM)

A549 cells were plated at a density of 1×10^5 in 35-mm glass bottom dishes (Matsunami, Osaka, Japan) at 37 °C in 1 mL of DMEM containing 10 % FBS for 24 h. pDNA was labeled with Cy5 by using a Label-IT reagent (Mirus, WI, USA) (labeling efficiency; 0.5 Cy5 / 100 base pair). Polyplexes at the *N/P* ratio of 7 were prepared with Cy5-labeled pDNA (2 μg) and polymer for 20 min at room temperature. The medium was replaced with Opti-MEM containing pDNA/polymer polyplexes. After incubation for 6 h, the medium was changed to DMEM containing 10 % FBS, and the cells were further incubated for following 1, 3, 6, 18, and 24 h. Polyplexes were observed by CLSM (ZEISS LSM 700, Carl Zeiss, Oberlochen, Germarny) equipped with a Plan-Apochromat 63×/1.40 Oil Ph3 M27 objective after staining acidic late endosomes and lysosomes with LysoTracker Green for 1 h and nuclei with Hoechst 33342 (Molecular Probes, Eugene, OR) for 15 min before each observation. The images were obtained by using manufacturer-specified laser excitation wavelengths and emission filter sets and processed with Zeiss Zen 2010 software at excitation wavelengths of 405 nm, 488 nm, and 633 nm for Hoechst 33342, LysoTracker Green, and Cy5, respectively.

Animal experiment

Animal studies were performed in accordance with the Guidelines for Animal Care and Use Committee at Kyushu University (Fukuoka, Japan). Male 4-week-old BALB/c nude mice were used in this study. Mice were inoculated with a dorsal, subcutaneous injection of 1×10^7 cells in 100 μL of Matrigel (BD Biosciences, Bedford, MA, USA) per animal. Tumors were allowed to grow to a mean diameter of approximately 8 mm. Introduction of complexes (100 μL) (pDNA, 100 μg/mL) into cancers or normal subcutaneous tissue was performed by a direct injection. After 24 h, mice were anesthetized and injected intraperitoneally with 0.2 mL of 15 mg/mL D-luciferin (potassium salt) (Promega, Madison, WI, USA) in Ringer's solution. Images were obtained using a cooled IVIS CCD camera (Xenogen, Alameda, CA, USA) and analyzed with Living Image software.

2.5 References

(1) Gene Therapy Clinical Trials Database, *J Gene Med* **2013,** John Wiley and Sons Ltd, wiley.com.

(2) Maguire, A. M.; Simonelli, F.; Pierce, E. A.; Pugh, E. N.; Mingozzi, F.; Bennicelli, J.; Banfi, S.; Marshall, K. A.; Testa, F.; Surace, E. M.; Rossi, S.; Lyubarsky, A.; Arruda, V. R.; Konkle, B.; Stone, E.; Sun, J. W.; Jacobs, J.; Dell'Osso, L.; Hertle, R.; Ma, J. X.; Redmond, T. M.; Zhu, X. S.; Hauck, B.; Zelenaia, O.; Shindler, K. S.; Maguire, M. G.; Wright, J. F.; Volpe, N. J.; McDonnell, J. W.; Auricchio, A.; High, K. A.; Bennett, J. *New Engl J Med* **2008**, *358*, 2240.

(3) Simonelli, F.; Maguire, A. M.; Testa, F.; Pierce, E. A.; Mingozzi, F.; Bennicelli, J. L.; Rossi, S.; Marshall, K.; Banfi, S.; Surace, E. M.; Sun, J. W.; Redmond, T. M.; Zhu, X. S.; Shindler, K. S.; Ying, G. S.; Ziviello, C.; Acerra, C.; Wright, J. F.; McDonnell, J. W.; High, K. A.; Bennett, J.; Auricchio, A. *Mol Ther* **2010**, *18*, 643.

(4) Cideciyan, A. V.; Hauswirth, W. W.; Aleman, T. S.; Kaushal, S.; Schwartz, S. B.; Boye, S. L.; Windsor, E. A. M.; Conlon, T. J.; Sumaroka, A.; Roman, A. J.; Byrne, B. J.; Jacobson, S. G. *New Engl J Med* **2009**, *361*, 725.

(5) Fischer, A.; Hacein-Bey-Abina, S.; Cavazzana-Calvo, M. *Nat Immunol* **2010**, *11*, 457.

(6) Chowdhury, E. H. *Expert Opin Drug Deliv* **2009**, *6*, 697.

(7) Smith, A. E. *Annu Rev Microbiol* **1995**, *49*, 807.

(8) Lee, M.; Kim, S. W. *Pharm Res-Dordr* **2005**, *22*, 1.

(9) Tian, J.; Xu, Z. L.; Smith, J. S.; Hofherr, S. E.; Barry, M. A.; Byrnes, A. P. *J Virol* **2009**, *83*, 5648.

(10) Huang, X.; Yang, Y. *Hum Gene Ther* **2009**, *20*, 293.

(11) Collins, S. A.; Guinn, B. A.; Harrison, P. T.; Scallan, M. F.; O'Sullivan, G. C.; Tangney, M. *Curr Gene Ther* **2008**, *8*, 66.

(12) Christie, R. J.; Nishiyama, N.; Kataoka, K. *Endocrinology* **2010**, *151*, 466.

(13) Park, T. G.; Jeong, J. H.; Kim, S. W. *Adv Drug Deliver Rev* **2006**, *58*, 467.

(14) Kang, J. H.; Toita, R.; Katayama, Y. *Biotechnol Adv* **2010**, *28*, 757.

(15) Mintzer, M. A.; Simanek, E. E. *Chem Rev* **2009**, *109*, 259.

(16) Liu, G.; Molas, M.; Grossmann, G. A.; Pasumarthy, M.; Perales, J. C.; Cooper, M. J.; Hanson, R. W. *J Biol Chem* **2001**, *276*, 34379.

(17) Wolfert, M. A.; Schacht, E. H.; Toncheva, V.; Ulbrich, K.; Nazarova, O.; Seymour, L. W. *Hum Gene Ther* **1996**, *7*, 2123.

(18) Yamagata, M.; Kawano, T.; Shiba, K.; Mori, T.; Katayama, Y.; Niidome, T. *Bioorgan Med Chem* **2007**, *15*, 526.

(19) Kwoh, D. Y.; Coffin, C. C.; Lollo, C. P.; Jovenal, J.; Banaszczyk, M. G.; Mullen, P.; Phillips, A.; Amini, A.; Fabrycki, J.; Bartholomew, R. M.; Brostoff, S. W.; Carlo, D. J. *Bba-Gene Struct Expr* **1999**, *1444*, 171.

(20) Densmore, C. L.; Orson, F. M.; Xu, B.; Kinsey, B. M.; Waldrep, J. C.; Hua, P.; Bhogal, B.; Knight, V. *Mol Ther* **2000**, *1*, 180.

(21) Neu, M.; Fischer, D.; Kissel, T. *J Gene Med* **2005**, *7*, 992.

(22) Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. *J Gene Med* **2001**, *3*, 362.

(23) Knorr, V.; Allmendinger, L.; Walker, G. F.; Paintner, F. F.; Wagner, E. *Bioconjugate Chem* **2007**, *18*, 1218.

(24) Godbey, W. T.; Wu, K. K.; Mikos, A. G. *J Control Release* **1999**, *60*, 149.

(25) Funhoff, A. M.; Van Nostrum, C. F.; Lok, M. C.; Kruijtzer, J. A. W.; Crommelin, D. J. A.; Hennink, W. E. *J Control Release* **2005**, *101*, 233.

(26) Oishi, M.; Kataoka, K.; Nagasaki, Y. *Bioconjugate Chem* **2006**, *17*, 677.

(27) van Steenis, J. H.; van Maarseveen, E. M.; Verbaan, F. J.; Verrijk, R.; Crommelin, D. J. A.; Storm, G.; Hennink, W. E. *J Control Release* **2003**, *87*, 167.

(28) Wakebayashi, D.; Nishiyama, N.; Yamasaki, Y.; Itaka, K.; Kanayama, N.; Harada, A.; Nagasaki, Y.; Kataoka, K. *J Control Release* **2004**, *95*, 653.

(29) Dubruel, P.; Christiaens, B.; Vanloo, B.; Bracke, K.; Rosseneu, M.; Vandekerckhove, J.; Schacht, E. *Eur J Pharm Sci* **2003**, *18*, 211.

(30) Sato, T.; Ishii, T.; Okahata, Y. *Biomaterials* **2001**, *22*, 2075.

(31) Lee, D.; Zhang, W.; Shirley, S. A.; Kong, X.; Hellermann, G. R.; Lockey, R. F.; Mohapatra, S. S. *Pharm Res-Dordr* **2007**, *24*, 157.

(32) Azzam, T.; Eliyahu, H.; Shapira, L.; Linial, M.; Barenholz, Y.; Domb, A. J. *J Med Chem* **2002**, *45*, 1817.

(33) Hosseinkhani, H.; Azzam, T.; Tabata, Y.; Domb, A. J. *Gene Ther* **2004**, *11*, 194.

(34) Koping-Hoggard, M.; Tubulekas, I.; Guan, H.; Edwards, K.; Nilsson, M.; Varum, K. M.; Artursson, P. *Gene Ther* **2001**, *8*, 1108.

(35) Lin, C.; Zhong, Z. Y.; Lok, M. C.; Jiang, X. L.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. *Bioconjugate Chem* **2007**, *18*, 138.

(36) Lin, C.; Zhong, Z. Y.; Lok, M. C.; Jiang, X. J.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. *J Control Release* **2007**, *123*, 67.

(37) Lin, C.; Zhong, Z. Y.; Lok, M. C.; Jiang, X. L.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. *J Control Release* **2006**, *116*, 130.

(38) Green, J. J.; Shi, J.; Chiu, E.; Leshchiner, E. S.; Langer, R.; Anderson, D. G. *Bioconjugate Chem* **2006**, *17*, 1162.

(39) Lynn, D. M.; Langer, R. *J Am Chem Soc* **2000**, *122*, 10761.

(40) Lim, Y. B.; Kim, S. M.; Suh, H.; Park, J. S. *Bioconjugate Chem* **2002**, *13*, 952.

(41) Wang, J.; Mao, H. Q.; Leong, K. W. *J Am Chem Soc* **2001**, *123*, 9480.

(42) Panyam, J.; Labhasetwar, V. *Adv Drug Deliver Rev* **2003**, *55*, 329.

(43) Danhier, F.; Feron, O.; Preat, V. *J Control Release* **2010**, *148*, 135.

(44) Glover, D. J.; Lipps, H. J.; Jans, D. A. *Nat Rev Genet* **2005**, *6*, 299.

(45) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. *Nat Rev Drug Discov* **2005**, *4*, 581.

(46) Nishiyama, N.; Kataoka, K. *Pharmacol Therapeut* **2006**, *112*, 630.

(47) Katayama, Y.; Fujii, K.; Ito, E.; Sakakihara, S.; Sonoda, T.; Murata, M.; Maeda, M. *Biomacromolecules* **2002**, *3*, 905.

(48) Kawamura, K.; Oishi, J.; Kang, J. H.; Kodama, K.; Sonoda, T.; Murata, M.; Niidome, T.; Katayama, Y. *Biomacromolecules* **2005**, *6*, 908.

(49) Asai, D.; Tsuchiya, A.; Kang, J. H.; Kawamura, K.; Oishi, J.; Mori, T.; Niidome, T.; Shoji, Y.; Nakashima, H.; Katayama, Y. *J Gene Med* **2009**, *11*, 624.

(50) Oishi, J.; Kawamura, K.; Kang, J. H.; Kodama, K.; Sonoda, T.; Murata, M.; Niidome, T.; Katayama, Y. *J Control Release* **2006**, *110*, 431.

(51) Kang, J. H.; Asai, D.; Kim, J. H.; Mori, T.; Toita, R.; Tomiyama, T.; Asami, Y.; Oishi, J.; Sato, Y. T.; Niidome, T.; Jun, B.; Nakashima, H.; Katayama, Y. *J Am Chem Soc* **2008**, *130*, 14906.

(52) Toita, R.; Kang, J. H.; Kim, J. H.; Tomiyama, T.; Mori, T.; Niidome, T.; Jun, B.; Katayama, Y. *J Control Release* **2009**, *139*, 133.

(53) Tomiyama, T.; Toita, R.; Kang, J. H.; Asai, D.; Shiosaki, S.; Mori, T.; Niidome, T.; Katayama, Y. *J Control Release* **2010**, *148*, 101.

(54) Kang, J. H.; Asai, D.; Yamada, S.; Toita, R.; Oishi, J.; Mori, T.; Niidome, T.; Katayama, Y. *Proteomics* **2008**, *8*, 2006.

(55) Varkouhi, A. K.; Scholte, M.; Storm, G.; Haisma, H. J. *J Control Release* **2011**, *151*, 220.

(56) Cho, Y. W.; Kim, J. D.; Park, K. *J Pharm Pharmacol* **2003**, *55*, 721.

(57) Davis, M. E. *Curr Opin Biotech* **2002**, *13*, 128.

(58) Lungwitz, U.; Breunig, M.; Blunk, T.; Gopferich, A. *Eur J Pharm Biopharm* **2005**, *60*, 247.

(59) Toita, R.; Kang, J. H.; Tomiyama, T.; Kim, C. W.; Shiosaki, S.; Niidome, T.; Mori, T.; Katayama, Y. *J Am Chem Soc* **2012**, *134*, 15410.

(60) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. *J Gene Med* **2005**, *7*, 657.

(61) Tomiyama, T.; Kang, J. H.; Toita, R.; Niidome, T.; Katayama, Y. *Cancer Sci* **2009**, *100*, 1532.

(62) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. *J Gene Med* **2005**, *7*, 657.

(63) Boussif, O.; Zanta, M. A.; Behr, J. P. *Gene Ther* **1996**, *3*, 1074.

(64) Kichler, A.; Leborgne, C.; Coeytaux, E.; Danos, O. *J Gene Med* **2001**, *3*, 135.

(65) Hama, S.; Akita, H.; Ito, R.; Mizuguchi, H.; Hayakawa, T.; Harashima, H. *Mol Ther* **2006**, *13*, 786.

(66) Masago, K.; Itaka, K.; Nishiyama, N.; Chung, U. I.; Kataoka, K. *Biomaterials* **2007**, *28*, 5169.

(67) Tamura, A.; Oishi, M.; Nagasaki, Y. *Biomacromolecules* **2009**, *10*, 1818.

(68) Uchida, H.; Miyata, K.; Oba, M.; Ishii, T.; Suma, T.; Itaka, K.; Nishiyama, N.; Kataoka, K. *J Am Chem Soc* **2011**, *133*, 15524.

(69) Yuba, E.; Nakajima, Y.; Tsukamoto, K.; Iwashita, S.; Kojima, C.; Harada, A.; Kono, K. *J Control Release* **2012**, *160*, 552.

(70) Eklund, A. *Anal Biochem* **1976**, *70*, 434.

(71) Kang, J. H.; Asai, D.; Toita, R.; Kitazaki, H.; Katayama, Y. *Carcinogenesis* **2009**, *30*, 1927.

CHAPTER 3

Stabilization of PKCα-responsive gene carrier via hydrophobic interaction

3.1 Introduction

Among the current major approaches used in non-viral gene delivery system, cationic polymers are widely used as artificial gene carrier with potential advantages over viral vector-mediated therapy, such as severe immune response, high production cost, and inconvenience of manufacturing and handling.[1-6]

These polymers can form polyplexes with anionic nucleic acids through the electrostatic interaction. However, the stability issues of those polyplexes have led researchers to develop alternative approaches for a further perspective. Thus, a number of strategies have been introduced to stabilize polyplexes. For example, polyplexes are modified with additional stabilizing features such as hydrophobic moieties,[7-12] chemical cross-linking such as disulfide cross-linking,[13-17] or PEG coating using di and/or tri-block copolymers.[18-22] In the case of PEG coating, PEG chains are sometimes introduced into polymer in detachable manner at particular conditions.[23-26] Such factors contribute to increase the colloidal stability and also prevent aggregation and interaction with serum proteins by a steric repulsion effect (**Figure 3.1**).[3]

Figure 3.1. Scheme of a block catiomer polyplex, showing a high stability in the extracellular medium and an efficient release of plasmid DNA (pDNA) in the intracellular compartment, was developed by controlling both the cationic charge and disulfide cross-linking densities of the backbone polycations.[15]

As mentioned in **Chapter 2**, a LPEI-peptide conjugate showed the clear-cut response to target PKCα both *in vitro* and *in vivo* (intratumoral injection), and showed a possibility as a cancer-specific alternative application in gene delivery. However, destabilization of polyplexes under physiological condition will be another obstacle for practical applications, especially under *in vivo* application such as the intravenous administration. As shown in **Figure 3.2**, a LPEI-peptide conjugate showed no expression in Balb/c mice after the intravenous administration, indicating that the degradation of pDNA in a blood flow was due to the dissociation of the polyplexes.[27]

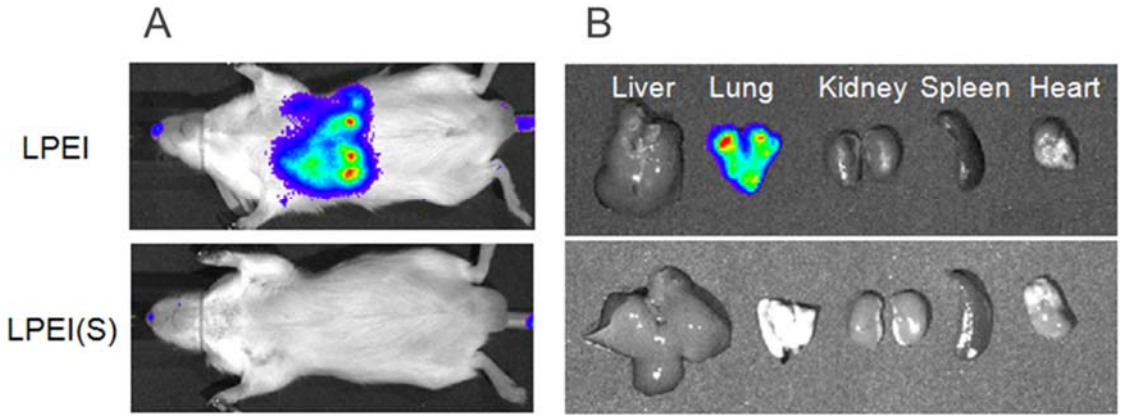


Figure 3.2. Images of luciferase activity of whole body of Balb/c mice (A) and isolated normal organs (B) 24 hours after intravenous injection of polyplexes (*N/P* ratio = 10, pDNA 20 μg).

Thus, I propose here a new carrier to stabilize polyplexes through the additional hydrophobic interaction. The carrier comprises a long alkyl spacer between the LPEI main chain and peptide. This simple modification was found to effectively stabilize the polyplexes, thereby leading to significantly improved PKCα-responsive gene expression (**Figure 3.3**).

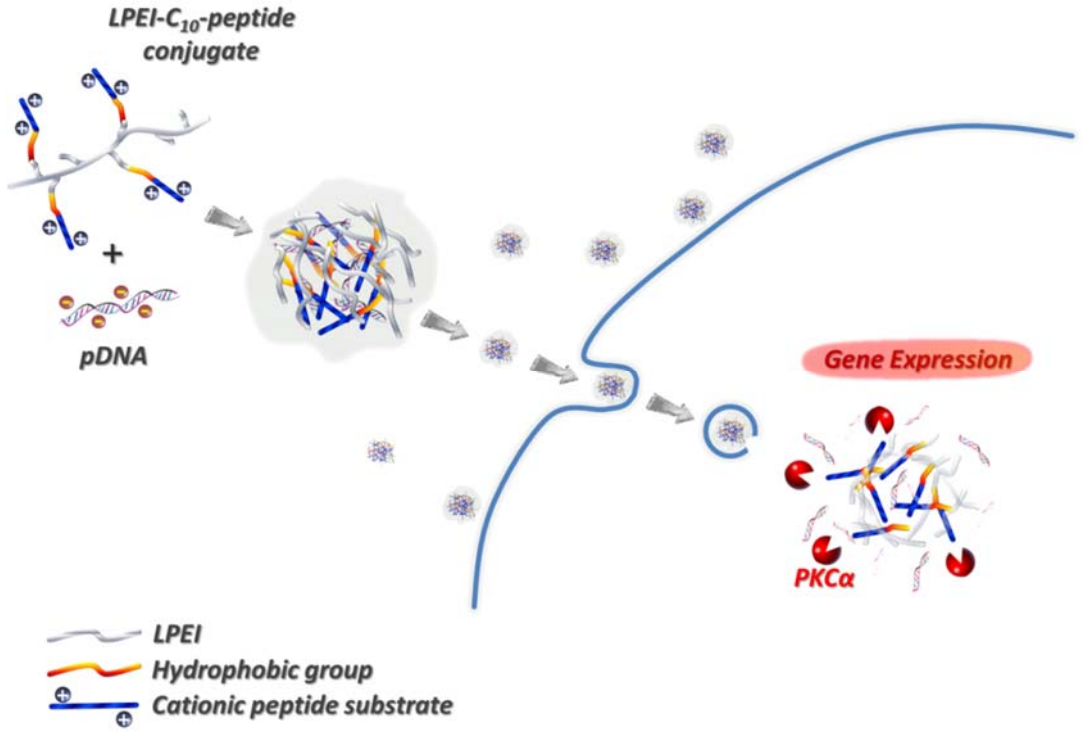
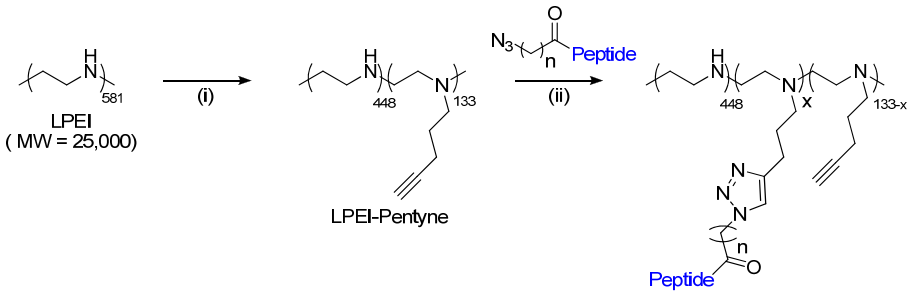


Figure 3.3. Concept of polyplexes stabilized by hydrophobic interaction for application in cancer-specific gene expression. The LPEI-C_{10}-peptide conjugate forms stable polyplexes with pDNA through electrostatic interactions between the anionic DNA strands and the cationic peptide substrate moieties, as well as through an additional hydrophobic interaction. Despite the enhanced stability of the polyplexes, the polyplexes were dissociated specifically in cancer cells responding to the phosphorylation reaction by PKCα.

3.2 Results and discussion

3.2.1 Reactivity of hydrophobically modified peptide substrate to PKCα

I previously used propionic acid linker for azide group modification on a terminus of the peptide substrate as mentioned in **Chapter 2**. Here I exchanged the linker for the much more hydrophobic undecanoic acid with the aim of achieving further stabilization of the polyplexes as shown in **Scheme 3.1**. I selected the *N*-terminus of the peptide for the introduction of the alkyl azide group, because we previously found that chemical modification of the *N*-terminus did not affect the overall reactivity of the peptide on the phosphorylation with PKCα. In contrast, chemical modification of the *C*-terminus of the peptide severely reduced the reactivity of the peptide. The azide group was modified on the *N*-terminus of the peptide in the last step of a solid phase peptide synthesis. Then, I assessed whether the reactivity of the resulting peptide substrate, C_{10}(S) [N_3-$(CH_2)_{10}$-CO-FKKQG**S**FAKKK-NH_2], toward PKCα is affected by the hydrophobic modification by using MALDI-TOF-MS. As shown in **Figure 3.4**, the phosphorylation of C_{10}(S) by PKCα was somewhat slower than that of C_2(S). However, after 30 min, the phosphorylation reaction of both C_2(S) and C_{10}(S) was almost saturated, indicating that the effect of the modification of the hydrophobic long alkyl chain on the reactivity of the peptide substrate to PKCα was not significant.



Scheme 3.1. Synthetic scheme for preparing hydrophobically modified LPEI-peptide conjugates.[a]
[a]Reagents and conditions: (i) 5-chloro-1-pentyne, DBU, and dry DMSO at 50°C; (ii) *N*-terminus azido-peptide, copper(II) pentahydrate, sodium ascorbate, and H_2O/ethanol = 1/1 at room temperature.

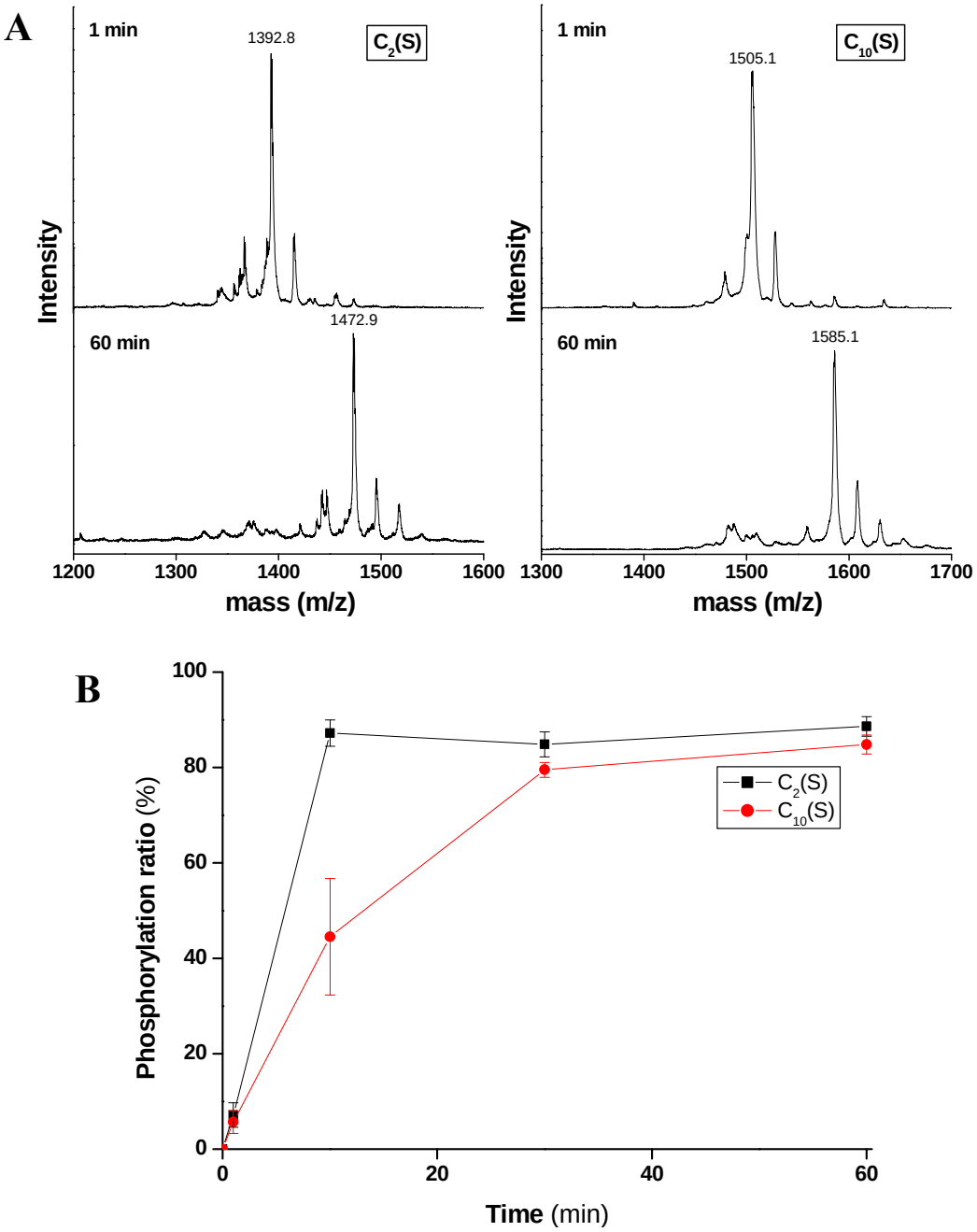


Figure 3.4. (A) Phosphorylation reactions of C_2(S) and C_{10}(S) in the presence of PKCα at 37 °C. (B) Time-dependent changes of the ratio of phosphorylation reactions. Phosphorylation ratios were identified by MALDI-TOF-MS analysis (n=3).

3.2.2 Reactivity of the hydrophobically modified LPEI-peptide polymer to PKCα

As mentioned in **Chapter 2**, the modification of the peptide substrates, C_{10}(S), on LPEI was performed via a two-step synthetic procedure as shown in **Scheme 3.1**. The contents of the peptide substrate were calculated from the peak area ratio between the protons of the phenyl groups in the peptide substrate (-CH-, δ=7.13 ppm) and the methylene protons of LPEI (-CH_2-, δ=2.85 ppm) by ^{1}H-NMR spectra in D_2O (**Figure 3.5**). These results showed that those of the contents of the peptide substrate in LPEI-C_{10}(S) and LPEI-C_{10}(A) were 10.8 mol % and 11.0 mol %, respectively (**Table 3.1**).

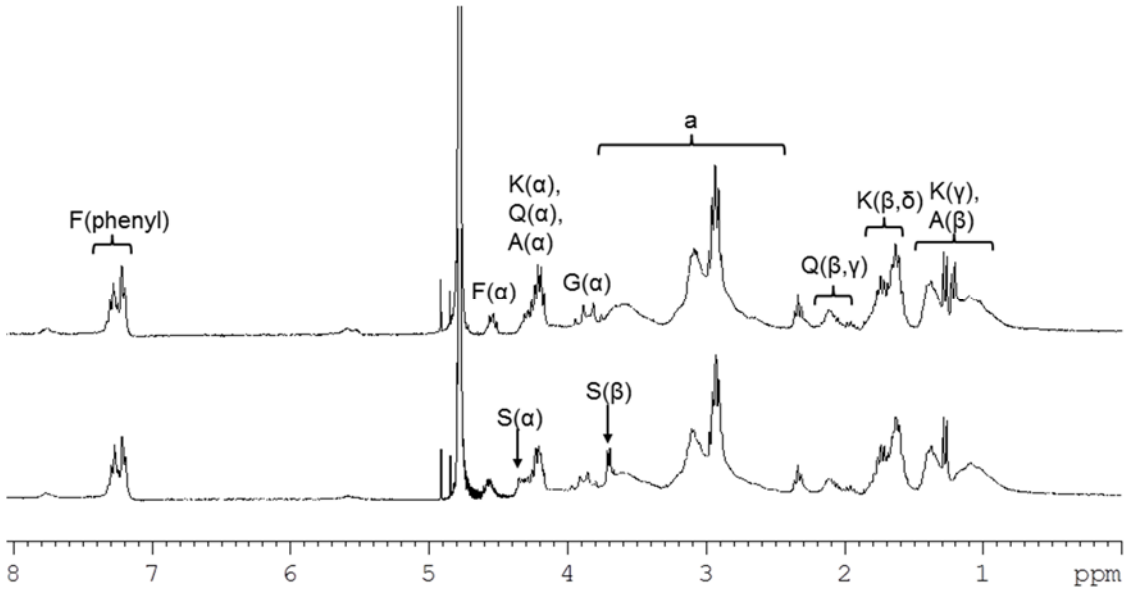


Figure 3.5. ^{1}H-NMR spectra of LPEI-C_{10}(A) (upper panel) and LPEI-C_{10}(S) (lower panel).

Table 3.1. Molecular parameters of carriers.

samples	n, alkyl chain length	peptide sequence	peptide content [a] / mol %	peptide no. / chain	M_w [b] / 10^4 g/mol
LPEI-C_2(S)	2	-FKKQG<u>S</u>FAKKK-NH_2	10.9	63	12.1
LPEI-C_2(A)	2	-FKKQG<u>A</u>FAKKK-NH_2	8.1	47	9.8
LPEI-C_{10}(S)	10	-FKKQG<u>S</u>FAKKK-NH_2	10.8	62	12.7
LPEI-C_{10}(A)	10	-FKKQG<u>A</u>FAKKK-NH_2	11.0	63	12.8

[a] Determined by ^{1}H-NMR spectra. [b] Calculated from peptide and pentyne contents and M_w of the parent LPEI.

The reactivity of the peptide substrate to PKCα after introducing into LPEI main chain was evaluated using a coupled enzyme assay. As shown in **Figure 3.6**, LPEI-C_{10}(A) showed no change in the absorption, while LPEI-C_{10}(S) showed steep decrease of the absorption resulting from the phosphorylation of the grafted peptide. Although the reaction rate of LPEI-C_{10}(S) is slower than that of the original peptide C_{10}(S), the reaction rate of LPEI-C_{10}(S) is surprisingly identical to that of LPEI-C_2(S). Thus, the effect of the long alkyl chain on the reactivity of the C_{10}(S) peptide was negligible after the C_{10}(S) peptide was modified onto the LPEI main chain. This is probably because the hydrophobic alkyl chain is hidden behind the peptide after modification onto the LPEI backbone.

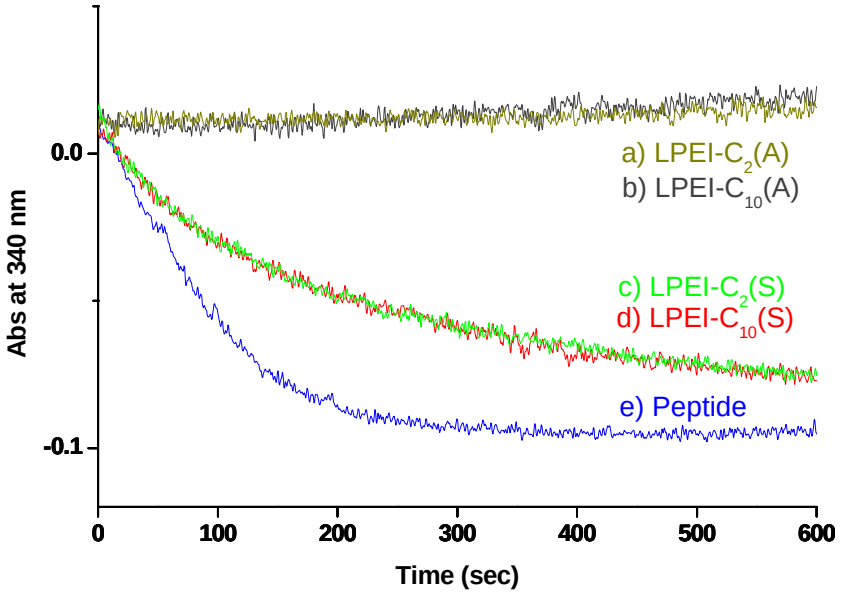


Figure 3.6. Reactivity of LPEI-peptide conjugates and original peptide (H-FKKQGSFAKKK-NH_2) toward PKCα monitored by a coupled enzyme assay. The phosphorylation reaction was identified from the decrease of absorbance at 340nm by NADH oxidation.

3.2.3 pDNA condensation ability of hydrophobically modified LPEI-peptide polymer

To verify the stoichiometry of the binding of the polymers to pDNA, an agarose gel electrophoresis assay was conducted with varying *N/P* ratios. The electrophoretic patterns for both LPEI-C_2(S) and LPEI-C_{10}(S) showed that free pDNA completely disappeared from the same *N/P* ratio of 2 (**Figure 3.7A**). Thus, the long alkyl chain of LPEI-C_{10}(S) seems not to interrupt the polyplexes formation.

The stoichiometry in the polyplexes formation was also verified by using an EtBr exclusion assay. EtBr produces a high fluorescence on interaction with nucleobases, whereas the fluorescence intensity significantly decreased by the inhibition of the intercalating processes of EtBr to DNA strand with the polyplexes formation causing condensation.[28] Thus, the distinct fluorescence intensity of intercalated EtBr is used to estimate the stoichiometry between polymer and DNA in the polyplexes

formation. As shown in **Figure 3.7B**, all four curves are almost superimposable and the relative fluorescence intensity (RFI) reached a plateau when the *N/P* ratio was 3. This result again shows that the long alkyl chain does not disturb the polyplexes formation. We previously found that, in LPEI-C_2(S), the cationic charges of the peptide substrate mainly contribute to the polyplexes formation while the charges of the LPEI main chain show negligible contribution to the condensation of pDNA probably due to the shielding of the cationic charges on the LPEI backbone by the bulky pentyne group.[27] The identical curves with LPEI-C_{10}(S) and LPEI-C_2(S) in **Figure 3.7B** showed that the cationic charges of the peptide also contributed mainly to the polyplexes formation in the case of LPEI-C_{10}(S). It is noteworthy that the anionic pDNA selectively binds to the cationic peptide of the carriers. This property is crucial for our transgene regulation system, which is specifically responsive to intracellular PKCα via phosphorylation reaction of the peptide substrate.

The size and size distribution of the polyplexes of LPEI-C_{10}(S)/pDNA or LPEI-C_2(S)/pDNA were investigated in 10 mM HEPES buffer at the *N/P* ratio of 7 and 10 (**Table 3.2**). The size of the polyplexes both of LPEI-C_{10}(S)/pDNA to LPEI-C_2(S)/pDNA were around 100 nm irrespective of the *N/P* ratios. The polydispersity index (PDI) of the both polyplexes was less than 0.21, which shows relatively narrow size distribution of these polyplexes. I then examined the stability of polyplexes against mixing with DMEM containing 10 % FBS by monitoring the dynamic light scattering (**Figure 3.8**). The diameter of the polyplexes from LPEI-C_{10}(S) is as stable as that of LPEI-C_2(S). The interparticle aggregation was not observed in the both polyplexes, when mixing with the medium. This result may indicate that the hydrophobic alkyl groups are not exposed to the surface of the polyplexes to induce interparticle aggregation.

Table 3.2. Diameter of LPEI-C_2(S) and LPEI-C_{10}(S) polyplexes in 10 mM HEPES buffer.

Samples	*N/P* ratio	Diameter / nm	PDI [a]
LPEI-C_2(S) polyplex	7	116.9 ± 1.3	0.178 ± 0.015
	10	116.2 ± 1.2	0.173 ± 0.013
LPEI-C_{10}(S) polyplex	7	98.1 ± 1.3	0.205 ± 0.019
	10	109.4 ± 1.1	0.161 ± 0.017

[a] PDI: polydisperse index

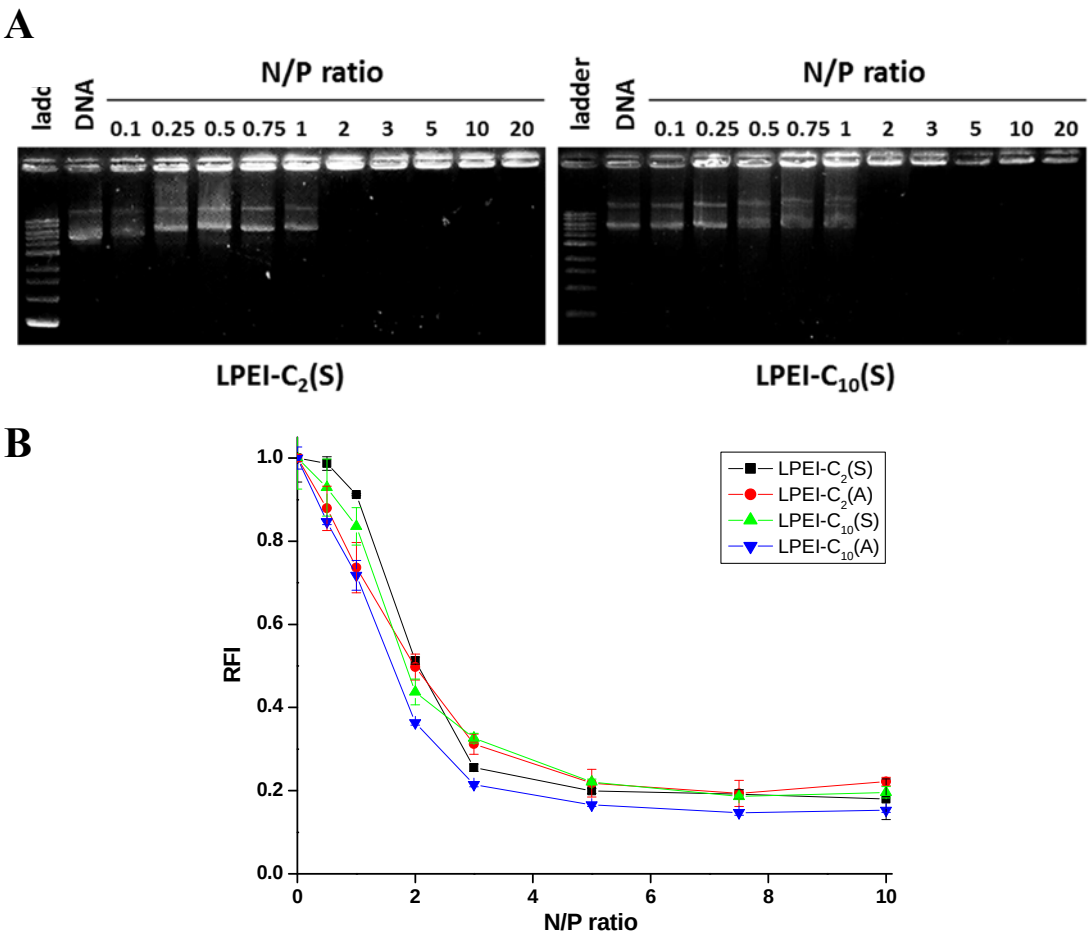


Figure 3.7. Polyplexes formation of the LPEI-peptide conjugate with pDNA. (A) Agarose gel electrophoresis of the conjugates and pDNA with varying *N/P* ratios. (B) EtBr exclusion assay of conjugates was performed by monitoring the distinct fluorescence intensity of intercalated EtBr (n=3).

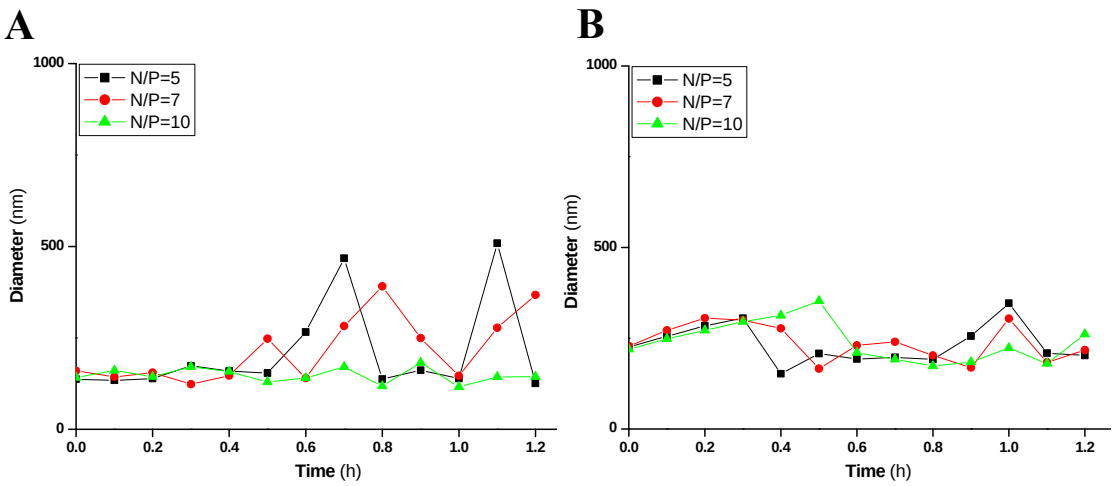


Figure 3.8. Time course of size change of (A) LPEI-C_2(S) and (B) LPEI-C_{10}(S) polyplexes in DMEM with 10% FBS.

3.2.4 Cytotoxicity and cellular uptake of hydrophobically modified polyplexes

The cytotoxicity of LPEI-C_2(S)/pDNA and LPEI-C_{10}(S)/pDNA was evaluated prior to *in vitro* studies. As shown in **Figure 3.9**, both LPEI-C_2(S) and LPEI-C_2(A) showed weak cytotoxicity at the *N/P* of 10, while the alkyl chain modified conjugates, LPEI-C_{10}(S) and LPEI-C_{10}(A) showed no detectable cytotoxicity. Thus, the hydrophobic alkyl modification somewhat reduced the cytotoxicity of the polymers.

To gain a better insight into the effect of an additional hydrophobic interaction, the cellular uptake of the polyplexes was monitored by using pDNA labeled with YOYO-1. As shown in **Figure 3.10**, the uptake of the LPEI-C_{10}(S) polyplex was higher than the LPEI-C_2(S) polyplex. The introduction of hydrophobic groups onto the polymer improved the intracellular uptake of the polyplexes probably because of the enhanced stability in the medium, which includes high concentrations of organic and inorganic salts and other ingredients. The enhanced stability of the polyplex will work to avoid undesirable transgene expression without phosphorylation in cytosol.

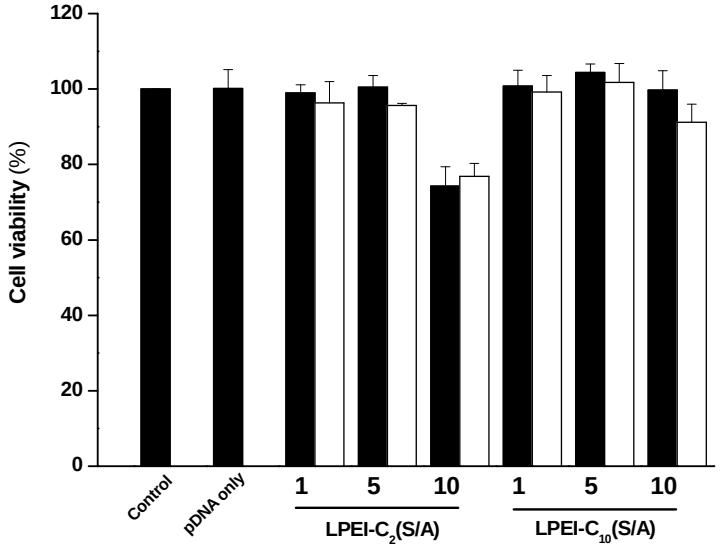


Figure 3.9. Cytotoxicity of polyplexes of LPEI-peptide conjugates for A549 cells (n=3). LPEI-C_2(S) and LPEI-C_{10}(S) are shown as filled bars, and LPEI-C_2(A) and LPEI-C_{10}(A) are shown as open bars.

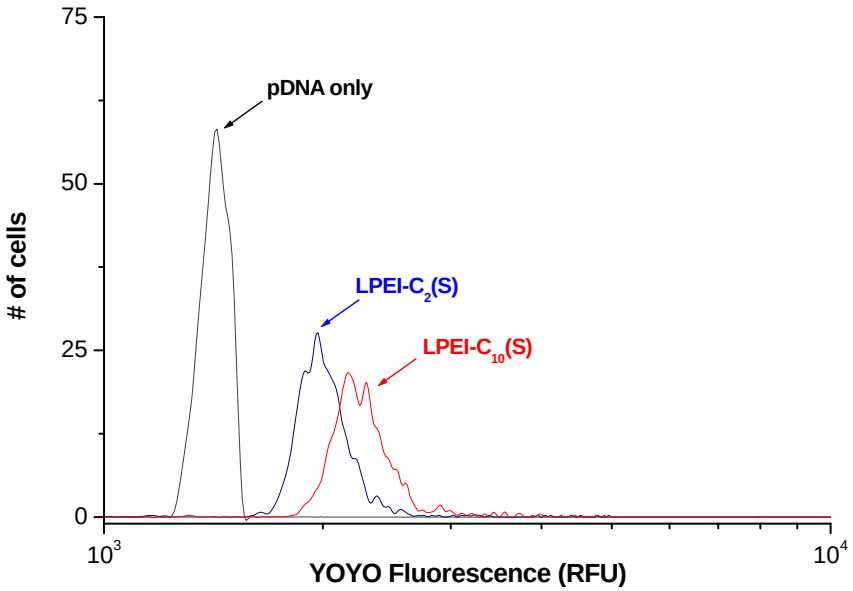


Figure 3.10. Cellular uptake of LPEI-C_2(S) and LPEI-C_{10}(S) polyplexes with YOYO-1-labeled pDNA in A549 cells. The *N/P* ratio of the polyplexes of the polymers was 7.

3.2.5 Intracellular trafficking of polyplexes

The intracellular trafficking of LPEI-C_2(S) and LPEI-C_{10}(S) polyplexes was compared by CLSM observation. I used here pDNA labeled with TAMRA and stained nuclei and late endosomes/lysosomes with Hoechst 33342 and LysoTracker Green, respectively. As shown in **Figure 3.11**, the fluorescent dots resulting from pDNA complexed with both LPEI-C_2(S) and LPEI-C_{10}(S) were observed in the cytoplasm with yellow and red, which results from the polyplex existing in the endosome and in the cytosol, respectively. In contrast, much smaller numbers of fluorescent dots were detected in the polyplex of LPEI-pentyne because of the weak complexation with pDNA.[27] These results indicated that LPEI-C_2(S) and LPEI-C_{10}(S) formed polyplexes which can be taken up by cells efficiently via endocytosis. However, the number of yellow and red dots was larger in LPEI-C_{10}(S)/pDNA than in LPEI-C_2(S)/pDNA. This results is consistent on the result in **Figure 3.10** showing enhanced effect of hydrophobic interaction on cellular uptake. The large number of red fluorescent dots of the polyplexes suggested that the buffering capacity of the LPEI main chain of LPEI-C_2(S) and LPEI-C_{10}(S) lead to escape from the endosome by the proton sponge effect.[29-32]

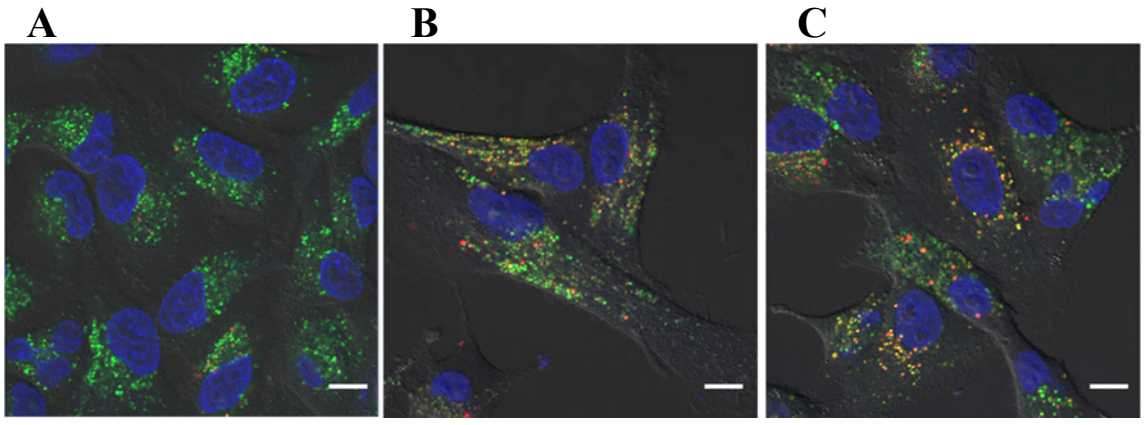


Figure 3.11. Intracellular distribution of (A) LPEI-pentyne, (B) LPEI-C_2(S), and (C) LPEI-C_{10}(S) polyplexes in A549 cells. pDNA was labeled with TAMRA (red). Late endosomes/lysosomes and the nuclei were stained with LysoTracker Green (green) and Hoechst 33342 (blue), respectively. The scale bar represents 20 μm.

3.2.6 Effect of hydrophobic interaction on regulation of transgene expression

The transgene regulation of the polyplexes was evaluated using luciferase-encoding pDNA in A549 cells, in which intracellular PKCα is known to be hyperactivated.[33-35] As shown in **Figure 3.12**, the gene expression of negative control polyplexes [LPEI-C_2(A) and LPEI-C_{10}(A)] was kept at a low level (< 10^7 RLU/mg protein), while the LPEI-C_2(S) and LPEI-C_{10}(S) polyplexes showed much higher gene expression, irrespective of the *N/P* ratios. This result clearly showed a suppression of gene expression in the negative control polyplexes and the PKCα-responsive gene expression in LPEI-C_2(S) and LPEI-C_{10}(S) polyplexes.

As for the effect of the alkyl chains, the LPEI-C_{10}(S) polyplexes showed a more than ten times higher gene expression than the LPEI-C_2(S) polyplexes at both the *N/P* ratios of 5 and 7. The higher gene expression in LPEI-C_{10}(S) should result from the improved cellular uptake in the transfection medium, which includes a high concentration of organic and inorganic salts as clarified in **Figure 3.10**. Despite the improved cellular uptake in the LPEI-C_{10}(S) polyplexes, the gene expression of the negative control polyplexes of LPEI-C_{10}(A) which also has the long alkyl chain showed a similar level as low as that of LPEI-C_2(A). This would be caused by the stabilization of the polyplexes via the enhanced hydrophobic interactions in LPEI-C_{10}(A). As a result of both the higher gene expression in LPEI-C_{10}(S) and the suppressed gene expression in LPEI-C_{10}(A), the LPEI(S)/LPEI(A) ratio [defined as the ratio of gene expression from LPEI(S) and that from LPEI(A)] was improved in LPEI-C_{10} from LPEI-C_2. Notably, LPEI-C_{10}(S) showed a 390 times higher gene expression than LPEI-C_{10}(A) at the *N/P* ratio 7.

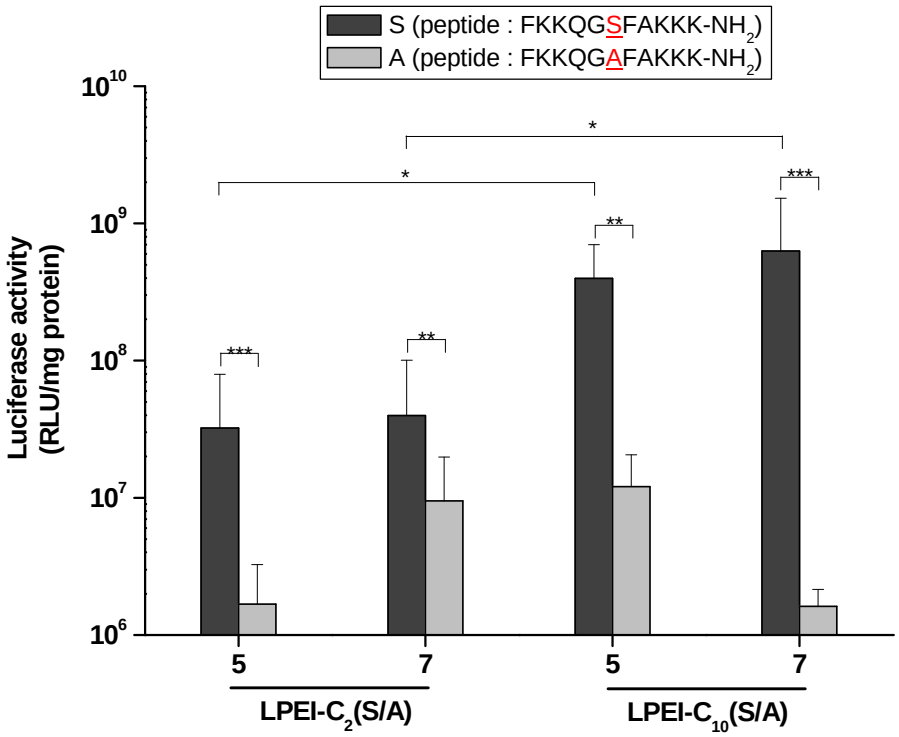


Figure 3.12. Transfection of polyplexes of LPEI-peptide conjugates with luciferase encoding pDNA into A549 cells (n=3). Data were expressed as the RLU/mg protein and the mean ± standard deviation from three different measurements (*p < 0.05, **p < 0.01, and ***p < 0.005).

3.3 Summary

Here, I demonstrated the ability of a hydrophobically modified LPEI-peptide conjugate as a cancer-specific gene carrier. A simple modification of a long alkyl chain as a spacer between the LPEI main chain and a substrate peptide enhanced the cellular uptake of the resultant polyplexes because of the effective stabilization of the polyplexes via hydrophobic interaction. It is important to note that the alkyl chain spacer did not affect the reactivity of the substrate peptide toward PKCα and the endosomal escaping ability. Because of these advantageous characteristics of the hydrophobically modified polyplexes, the polyplexes showed excellent performance in the cancer-signal responsive gene expression. Thus, the hydrophobically modified LPEI-peptide conjugate represents a promising carrier for cancer-specific gene therapy especially when cytotoxic suicide genes are employed.

3.4 Experimental section

Synthesis of the hydrophobically modified azido PKCα-specific peptide substrate

The peptide, FKKQG**S**FAKK, was prepared by using an automatic peptide synthesizer, according to standard Fmoc-chemistry procedures as mentioned in **Chapter 2**. To modify the hydrophobic groups on the peptide substrate, 11-Azidoundecanoic acid was synthesized from 11-Bromoundecanoic acid by addition of two equivalents of sodium azide for 1 day at room temperature in 1:1 DMF/DMSO solution. 11-Azidoundecanoic acid was then reacted with the *N*-terminus of the peptide in the presence of the coupling reagents. After completion of the reaction, the peptide was cleaved from the resin and was purified by reverse-phase liquid chromatography. The obtained peptide substrate, N_3-$(CH_2)_{10}$-CO-FKKQG**S**FAKK-NH_2 [C_{10}(S)] was identified by MALDI-TOF-MS. N_3-$(CH_2)_{10}$-CO-FKKQG**A**FAKK-NH_2 [C_{10}(A)] as a control peptide substrate was synthesized in the same manner.

Synthesis of the hydrophobically modified LPEI-peptide conjugate

The hydrophobically modified LPEI-peptide conjugates, LPEI-C_{10}(S) and LPEI-C_{10}(A), were synthesized by following a two-step synthetic procedure as described in **Chapter 2**. The contents of the peptide substrate were determined by ^{1}H-NMR spectra. LPEI-C_2(S) and LPEI-C_2(A) were synthesized in the same manner.

Phosphorylation assay of azido peptide substrate

The phosphorylation of C_2(S) and C_{10}(S) responding to PKCα was carried out in 100 μL buffer [10 mM HEPES, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.0 μg/mL diacylglycerol (DAG), 2.5 μg/mL phosphatidylserine (PS), and 200 μM ATP] containing 30 μM azido peptide substrate and 0.1 μg/mL PKCα. The phosphorylation was allowed to proceed for 1 h at 37 °C and then the resulting solutions were analyzed by MALDI-TOF-MS [34].

Phosphorylation assay of polymer using a coupled enzyme assay

The phosphorylation profiles of LPEI-C_2(S/A) and LPEI-C_{10}(S/A) were examined using a coupled enzyme assay.[35,36] The reaction was performed in 20 mM HEPES buffer [200 μM ATP, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.0 μg/mL DAG, 2.5 μg/mL PS, 0.3 mM nicotinamide adenine dinucleotide (NADH), 1 mM phosphoenolpyruvate, 10 U/μL LDH, and 4 U/μL pyruvate kinase] containing 30 μM polymer at 25 °C. Monitoring of NADH consumption was initiated by adding 1.1 ng/μL PKCα and detected with a UV/Vis spectrophotometer (UV-2550; Shimadzu, Tokyo, Japan) equipped with an SPR-8 temperature controller (Shimadzu) at 340 nm.

Agarose gel electrophoresis

Polyplexes at various *N/P* ratios were prepared with pDNA (0.2 μg) and different concentrations of polymer in 10 μL HEPES buffer (100 mM, pH 7.3) for 15 min at room temperature. The formation of the polyplexes was analyzed by 1% agarose gel electrophoresis at 100V for 30 min.

Ethidium bromide (EtBr) exclusion assay

Five micro liters of pDNA (0.1 μg/μL) was prepared with 1.25 μL of EtBr (0.1 μg/μL) for 5min. Polyplexes at various *N/P* ratios were prepared by adding different concentrations of polymer for 15 min and the volume of each sample was adjusted to 100 μL with 10 mM HEPES. Assays were performed at room temperature in the dark. Fluorescence measurements of each sample were performed at 25 °C using a multilabel counter ARVO (Wallac Incorporated, Turku, Finland). Excitation and emission wavelengths were 530 nm and 590 nm, respectively. The relative fluorescence intensity (RFI) was determined by using the following equation : $RFI = (F_{obs} - F_e)/(F_0 - F_e)$, where F_{obs}, F_e and F_0 are the fluorescence intensities of the polyplexes at each *N/P* ratio, back ground (EtBr only), and naked pDNA, respectively.

Dynamic light scattering (DLS) measurement

Polyplexes at various *N/P* ratios were prepared with pDNA and different concentrations of polymer by mixing at room temperature for 20 min. The final concentration of pDNA was adjusted to 20 μg/mL in 10 mM HEPES buffer or Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen Co., Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS). The diameters of the polymer/pDNA polyplexes were measured by a Zetasizer (Malvern Instruments, Worcestershire, UK) with the He/Ne laser at a detection angle of 173 ° at 25 °C.

Cell culture

A549 cells were cultured in DMEM containing 10 % FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Gibco). The cell was harvested in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

Cellular uptake of polyplexes

The pDNA was fluorescently labeled with the intercalating nucleic acid dye YOYO-1 iodide (diluted from 1 mM stock solution in DMSO, Molecular Probes) for uptake analysis.[37] 500 μL of pDNA (0.1 mg/mL) was combined with 100 μL of TAE buffer and 400 μL of 10 μL YOYO-1 in TE buffer in a microcentrifuge tube. The solution was mixed for at least 1 h at room temperature in the dark wrapped in foil and stored at -20 °C.

200 μL Aliquots of growing A549 cell suspension (30,000 cells) were seeded into a 48-well plate in DMEM containing 10 % FBS. After incubation for 24 h, the medium in each well was replaced with Opti-MEM (Gibco) containing the YOYO-1-labeled pDNA/polymer polyplexes at the *N/P* ratio 7, and incubated for 2 h. Estimation of cellular uptake was conducted by a Tali™ Image-Based Cytometer (Invitrogen).

Confocal laser scanning microscope (CLSM)

A549 cells were plated at a density of 1×10^5 in 35-mm glass bottom dishes (Matsunami, Osaka, Japan) at 37 °C in 1 mL of DMEM containing 10 % FBS for 24 h. pDNA was labeled with tetramethylrhodamine (TAMRA) by using a Label-IT reagent (Mirus, WI, USA) (labeling efficiency; 1 TAMRA / 100 base pair). Polyplexes at the *N/P* ratio of 7 were prepared with TAMRA-labeled pDNA (2 μg) and polymer for 20 min at room temperature. The medium was replaced with Opti-MEM containing pDNA/polymer polyplexes. After incubation for 6 h, the medium was changed to DMEM containing 10 % FBS, and the cells were further incubated for 18 h. Polyplexes were observed by CLSM (ZEISS LSM 700, Carl Zeiss, Oberlochen, Germarny) equipped with a Plan-Apochromat 63×/1.40 Oil Ph3 M27 objective after staining acidic late endosomes and lysosomes with LysoTracker Green for 1 h and nuclei with Hoechst 33342 (Molecular Probes, Eugene, OR) for 15 min before each observation. The images were obtained by using manufacturer-specified laser excitation wavelengths and emission filter sets and processed with Zeiss Zen 2010 software at excitation wavelengths of 405 nm, 488 nm, and 555 nm for Hoechst 33342, LysoTracker Green, and TAMRA, respectively.

Transfection study

A549 cells were plated at a density of 3×10^4 in 48-well plates at 37 °C in DMEM containing 10 % FBS for 24 h. Polyplexes at various *N/P* ratios were prepared with pDNA (1 μg) and different concentrations of polymer for 20 min at room temperature. The medium in each well was replaced with Opti-MEM containing pDNA/polymer polyplexes at various *N/P* ratios. After incubation for 4 h, the medium was changed to DMEM containing 10 % FBS, and the cells were further incubated for 20 h. The cultured cells were then scraped and lysed in 100 μL of lysis buffer (20 mM Tris-HCl, pH 7.4, 0.05 % Triton-X 100, and 2 mM EDTA). A 10-μL aliquot of the lysate solution was used for measuring chemiluminescence in a MiniLumat LB 9506 (EG & G Berthold, Wildbad, Germany) directly after mixing with 40 μL of the luciferin substrate. The results are presented as relative luminescence units (RLU)/mg total protein.

3.5 References

(1) Davis, M. E. *Curr Opin Biotech* **2002**, *13*, 128.

(2) Park, T. G.; Jeong, J. H.; Kim, S. W. *Adv Drug Deliver Rev* **2006**, *58*, 467.

(3) Christie, R. J.; Nishiyama, N.; Kataoka, K. *Endocrinology* **2010**, *151*, 466.

(4) de Ilarduya, C. T.; Sun, Y.; Duezguenes, N. *Eur J Pharm Sci* **2010**, *40*, 159.

(5) Kim, T. I.; Kim, S. W. *React Funct Polym* **2011**, *71*, 344.

(6) Miyata, K.; Nishiyama, N.; Kataoka, K. *Chem Soc Rev* **2012**, *41*, 2562.

(7) Muller, M.; Kessler, B.; Richter, S. *Langmuir* **2005**, *21*, 7044.

(8) Voets, I. K.; de Keizer, A.; Stuart, M. A. C.; Justynska, J.; Schlaad, H. *Macromolecules* **2007**, *40*, 2158.

(9) Kim, H. J.; Ishii, A.; Miyata, K.; Lee, Y.; Wu, S. R.; Oba, M.; Nishiyama, N.; Kataoka, K. *J Control Release* **2010**, *145*, 141.

(10) Akagi, T.; Watanabe, K.; Kim, H.; Akashi, M. *Langmuir* **2010**, *26*, 2406.

(11) Yuan, X. F.; Harada, A.; Yamasaki, Y.; Kataoka, K. *Langmuir* **2005**, *21*, 2668.

(12) Philipp, A.; Zhao, X. B.; Tarcha, P.; Wagner, E.; Zintchenko, A. *Bioconjugate Chem* **2009**, *20*, 2055.

(13) Lee, Y.; Mo, H.; Koo, H.; Park, J. Y.; Cho, M. Y.; Jin, G. W.; Park, J. S. *Bioconjugate Chem* **2007**, *18*, 13.

(14) Christie, R. J.; Tadiello, C. J.; Chamberlain, L. M.; Grainger, D. W. *Bioconjugate Chem* **2009**, *20*, 476.

(15) Miyata, K.; Kakizawa, Y.; Nishiyama, N.; Harada, A.; Yamasaki, Y.; Koyama, H.; Kataoka, K. *J Am Chem Soc* **2004**, *126*, 2355.

(16) Matsumoto, S.; Christie, R. J.; Nishiyama, N.; Miyata, K.; Ishii, A.; Oba, M.; Koyama, H.; Yamasaki, Y.; Kataoka, K. *Biomacromolecules* **2009**, *10*, 119.

(17) Neu, M.; Germershaus, O.; Behe, M.; Kissel, T. *J Control Release* **2007**, *124*, 69.

(18) Fukushima, S.; Miyata, K.; Nishiyama, N.; Kanayama, N.; Yamasaki, Y.; Kataoka, K. *J Am Chem Soc* **2005**, *127*, 2810.

(19) Oishi, M.; Kataoka, K.; Nagasaki, Y. *Bioconjugate Chem* **2006**, *17*, 677.

(20) Miyata, K.; Oba, M.; Kano, M. R.; Fukushima, S.; Vachutinsky, Y.; Han, M.; Koyama, H.; Miyazono, K.; Nishiyama, N.; Kataoka, K. *Pharm Res-Dordr* **2008**, *25*, 2924.

(21) Lee, H. J.; Kim, S. E.; Kwon, I. K.; Park, C.; Kim, C.; Yang, J.; Lee, S. C. *Chem Commun* **2010**, *46*, 377.

(22) Cheng, C.; Convertine, A. J.; Stayton, P. S.; Bryers, J. D. *Biomaterials* **2012**, *33*, 6868.

(23) Sethuraman, V. A.; Na, K.; Bae, Y. H. *Biomacromolecules* **2006**, *7*, 64.

(24) Walker, G. F.; Fella, C.; Pelisek, J.; Fahrmeir, J.; Boeckle, S.; Ogris, M.; Wagner, E. *Mol Ther* **2005**, *11*, 418.

(25) Knorr, V.; Allmendinger, L.; Walker, G. F.; Paintner, F. F.; Wagner, E. *Bioconjugate Chem* **2007**, *18*, 1218.

(26) Oumzil, K.; Khiati, S.; Grinstaff, M. W.; Barthelemy, P. *J Control Release* **2011**, *151*, 123.

(27) Toita, R.; Kang, J. H.; Tomiyama, T.; Kim, C. W.; Shiosaki, S.; Niidome, T.; Mori, T.; Katayama, Y. *J Am Chem Soc* **2012**, *134*, 15410.

(28) Tsuchiya, A.; Kang, J. H.; Asai, D.; Mori, T.; Niidome, T.; Katayama, Y. *J Control Release* **2011**, *155*, 40.

(29) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. *Nat Rev Drug Discov* **2005**, *4*, 581.

(30) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. *J Gene Med* **2005**, *7*, 657.

(31) Mintzer, M. A.; Simanek, E. E. *Chem Rev* **2009**, *109*, 259.

(32) Whitehead, K. A.; Langer, R.; Anderson, D. G. *Nat Rev Drug Discov* **2009**, *8*, 129.

(33) Kang, J. H.; Asai, D.; Kim, J. H.; Mori, T.; Toita, R.; Tomiyama, T.; Asami, Y.; Oishi, J.; Sato, Y. T.; Niidome, T.; Jun, B.; Nakashima, H.; Katayama, Y. *J Am Chem Soc* **2008**, *130*, 14906.

(34) Kang, J. H.; Asai, D.; Toita, R.; Kitazaki, H.; Katayama, Y. *Carcinogenesis* **2009**, *30*, 1927.

(35) Tomiyama, T.; Kang, J. H.; Toita, R.; Niidome, T.; Katayama, Y. *Cancer Sci* **2009**, *100*, 1532.

(36) Toita, R.; Kang, J. H.; Kim, J. H.; Tomiyama, T.; Mori, T.; Niidome, T.; Jun, B.; Katayama, Y. *J Control Release* **2009**, *139*, 133.

(37) Doyle, S. R.; Chan, C. K. *Genet Vaccines Ther* **2007**, *5*, 11.

CHAPTER 4

Regulation of PKCα activity and their effect on reversal of the resistance to drug in MCF-7/ADR cells

4.1 Introduction

Multidrug resistance (MDR) of cancer cells is a major factor that limits the effectiveness of cancer chemotherapy. Although MDR has a number of causes in a failure of chemotherapy, a widely studied mechanism of resistance is associated with the overexpression of the ATP-binding cassette (ABC) transporter superfamily, resulting in increased efflux of chemotherapeutic agents from cells. Among 48 human ABC transporter superfamily, a well-characterized P-glycoprotein (Pgp) is highly implicated with the reduction of intracellular drug levels below their therapeutic threshold and the consequent decrease of their toxic effects.[1-7]

Pgp is a 170 kDa ATP-dependent transmembrane protein, which is encoded by the ABCB1 (or MDR1) gene. This protein structurally contains two similar halves with an inward-facing conformation, consisting of 12 transmembrane domains (TMDs) and 2 nucleotide-binding domains (NBDs). The TMDs, presenting pseudo two-fold molecular symmetry spanning ~136 Å perpendicular to and ~70 Å in the plane of the membrane bilayer, recognize the wide range of substrates and subsequently transport these substrates to the outer of membrane bilayer, resulting in structural changes with an outward-facing conformation caused by ATP hydrolysis in the NBDs as shown in **Figure 4.1**.[4,8-12]

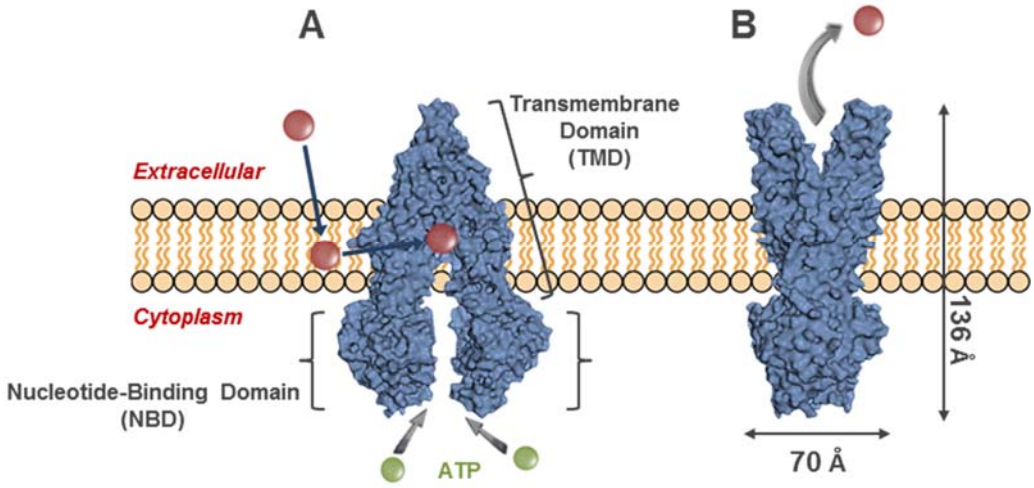


Figure 4.1. Model mechanism of substrate transport by Pgp. (A) Substrate partitions into the bilayer from outside of the cell to the inner leaflet and enters the internal drug-binding pocket through an open portal in the inward-facing conformation. (B) ATP binds to the NBDs causing a large conformational change presenting the substrate and drug-binding site(s) to the outer leaflet and/or extracellular space.[10]

A significant contribution of Pgp to the efflux of endogenous and exogenous molecules, which leads to drug resistance, provides an alternative rationale for developing targeted therapies against this membrane transporter.[13] Since inhibitors and modulators of Pgp were firstly identified in 1980's, inhibiting of Pgp has been extensively investigated as a strategy for reversal of multidrug resistance.[5,14] Initially, the first-generation Pgp inhibitors (e.g. verapamil, cyclosporin, and tamoxifen) were used as substrates of Pgp, competing with cellular efflux of therapeutic drugs, but it was limited by unacceptable systemic toxicity in clinical trials.[15-22] The second-generation Pgp inhibitors (e.g. dexverapamil, dexniguldipine, valspodar (PSC 833), and biricodar (VX-710)), which were structurally modified from the first-generation agents, had reduced primary toxicity, but it had the potential to change the pharmacokinetics of conventional drugs and inhibit metabolism of cytotoxic drugs.[23-32] Currently, the next third-generation Pgp inhibitors have been suggested by using structure-activity relationships and combinatorial chemistry to overcome the limitations of the second-generation Pgp inhibitors. These third-generation Pgp inhibitors (e.g. tariquidar (XR9576), zosuquidar (LY335979), and laniquidar (R101933)) are in various stages of clinical trials, showing a high potency and specificity for the Pgp transporter (**Figure 4.2**).[5,14,33-42]

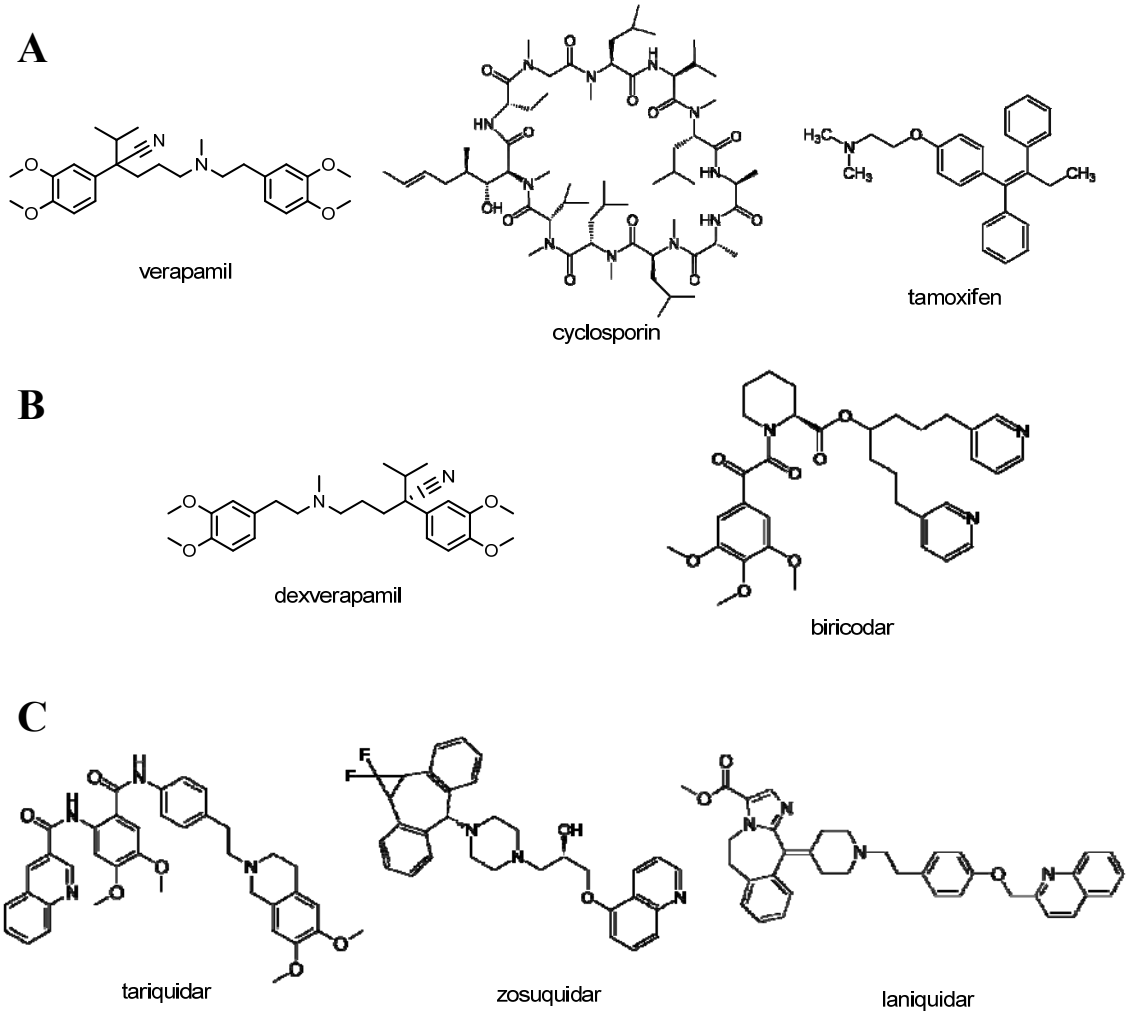


Figure 4.2. Representative structure of (A) first-generation Pgp inhibitors, (B) second-generation Pgp inhibitors, and (C) third-generation Pgp inhibitors.

Despite the distinct rationale for the direct inhibition and modulation of Pgp in cancer chemotherapy, there still remain many obstacles such as safety concerns and pharmacokinetic changes of therapeutic drugs in clinical trials. Thus, alternative approaches have been extensively conducted, offering a promising potential to overcome multidrug resistance by using new chemotherapeutic agents designed as analog of therapeutic compounds,[43-45] prodrugs,[46-48] Pgp-specific therapeutic antibodies,[49-51] and nanoparticle-based drug delivery systems (e.g. liposomes,[52-54] polymeric nanoparticles,[55-58] micelles,[59-61] inorganic nanoparticles,[62-65] and hybrid

nanoparticles).[66-68] These diverse strategies are concurrently emerging as promising candidates for effective cancer treatment, however, the toxicology of long-term exposure to drug or nanomaterials in human still need to be fully studied and evaluated.[69-71]

As previously mentioned in **Chapter 1**, many cellular processes are regulated by signal transduction, resulted in tight and reversible controls of protein phosphorylation via enzyme activities, protein kinases and phosphatases. The overexpression of Pgp has been implicated as a prominent role in human MDR cells, and one potential regulatory mechanism of Pgp activity is known to be regulated by protein phosphorylation, particularly induced by the activated protein kinase C (PKC) isoforms.[72-74] Many reports of drug accumulation in human MDR cells have been suggested that increased activities and levels of PKC isoforms, mainly for PKCα, phosphorylate specific serine residues in the linker region of Pgp, inducing alterations of Pgp activity with increased their ATPase activity and drug efflux (**Figure 4.3**).[75-79]

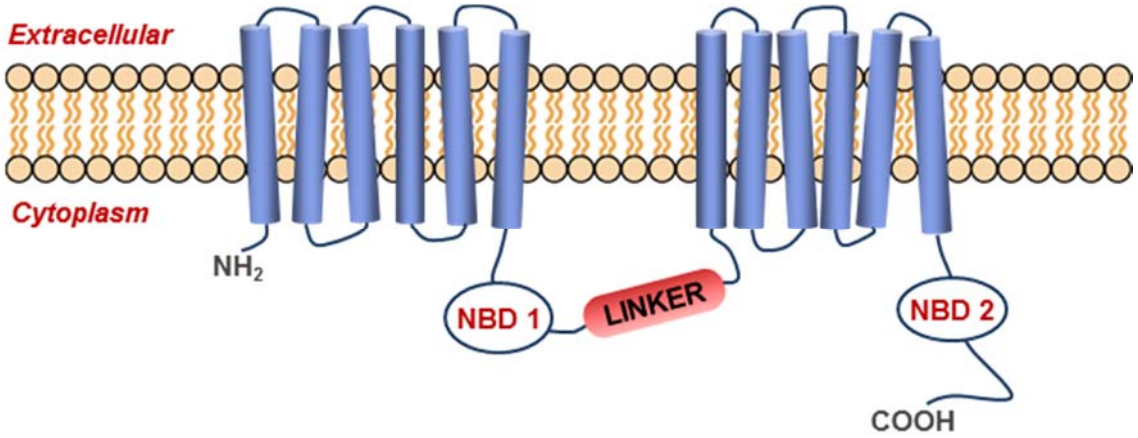


Figure 4.3. Schematic structural organization of P-glycoprotein. Each half contains 6 transmembrane α-helices involved in chemotherapeutic drug efflux, and nucleotide binding domain 1(NBD1) and NMD 2, containing an ATP-binding site. . The two halves are separated by linker region which is phosphorylated at several sites by protein kinase C.[79,80]

In this chapter, I investigate whether Pgp-mediated drug efflux is modulated by the inhibition of PKCα activity in MDR cells. To assess the levels of PKCα activity, a PKCα-specific peptide substrate (Alphatomega; FKKQG**S**FAKKK-NH_2) was used to

compare with their phosphorylation levels in drug-sensitive MCF-7 cells and drug-resistant MCF-7/ADR cells, which are well-characterized human cancer cell lines for multidrug resistance.[81-83] Here, I investigated the resistance to therapeutic drug, doxorubicin, in both cells and the activity of PKCα was modulated by PKC inhibitor, Ro-31-7549, which has great specificity toward PKCα (**Figure 4.4**).[84-86] In addition, the inhibition of PKCα exhibited increased drug accumulation in resistant cells, providing evidence that PKCα has a prominent role in the regulation of drug efflux.

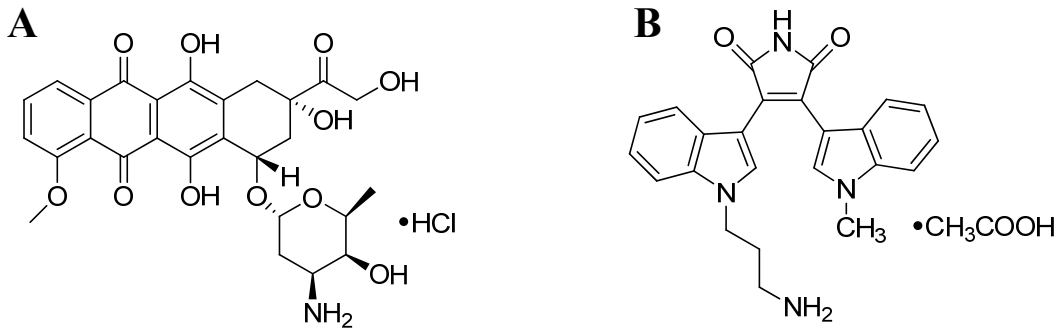


Figure 4.4. Structure of (A) doxorubicin and (B) protein kinase C inhibitor (Ro-31-7549). (A) antitumor antibiotic agent that inhibits DNA topoisomerase II. DNA intercalator that inhibits nucleic acid synthesis and induces apoptosis. (B) selective PKC inhibitor that acts at the ATP binding site of PKC.

4.2 Results and discussion

4.2.1 Resistance to doxorubicin in human breast cancer cells

In order to investigate the resistance to therapeutic drug, doxorubicin, in drug-sensitive MCF-7 and resistant MCF-7/ADR cells, I first compared the cell viability by using WST-8 assay in the presence of various concentration of doxorubicin. Both cells were exposed to increasing concentrations of doxorubicin (0.01 to100 nM) for 72 h (**Figure 4.5**). As expected, MCF-7 cells showed quite high sensitive to doxorubicin, with an IC_{50} of < 0.1 nM, whereas MCF-7/ADR cells showed ~100 times higher resistance to doxorubicin than MCF-7 cells, with IC_{50} of > 10 nM.

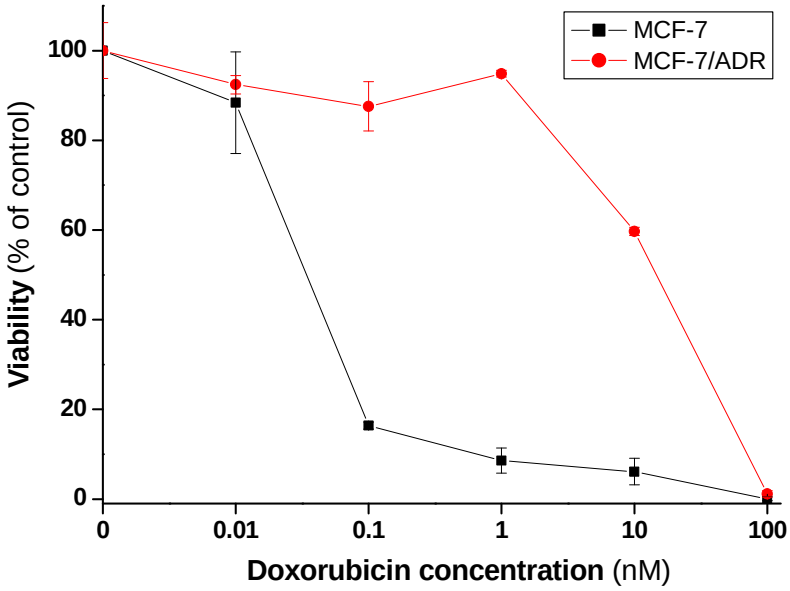


Figure 4.5. Cell viability with doxorubicin in MCF-7 (sensitive) and MCF-7/ADR (resistant) cells.

4.2.2 Regulation of PKCα activity in sensitive/resistant cancer cells by PKC inhibitor

The activity of PKCα was conveniently confirmed by phosphorylation levels of a PKCα-specific peptide substrate (Alphatomega; FKKQG**S**FAKKK-NH_2) with both MCF-7 and MCF-7/ADR cell lysates at a 200 μg/mL protein concentration. In addition, the inhibition of PKCα activity was identified with the addition of increasing concentration of PKC inhibitor (Ro-31-7549). As shown in **Figure 4.6**, the phosphorylation of the PKCα-specific peptide substrate in MCF-7/ADR cells showed relatively higher (84%) compared with MCF-7 (63%), which reflects the higher basal activity of PKCα in drug-resistant MCF-7/ADR cells than that in sensitive MCF-7 cells.[87,88] The addition of Ro-31-7549 led to a decrease of the phosphorylation level in both MCF-7 (63% to 5%) and MCF-7/ADR (84% to 31%) cells, respectively. Compared with MCF-7 cells, the PKCα activity in MCF-7/ADR cells was less sensitive against Ro-31-7549, showing 20-fold higher IC_{50} values for Ro-31-7549 (IC_{50} values: 0.76 μM in MCF-7 cells, 15.06 μM in MCF-7/ADR cells, respectively).

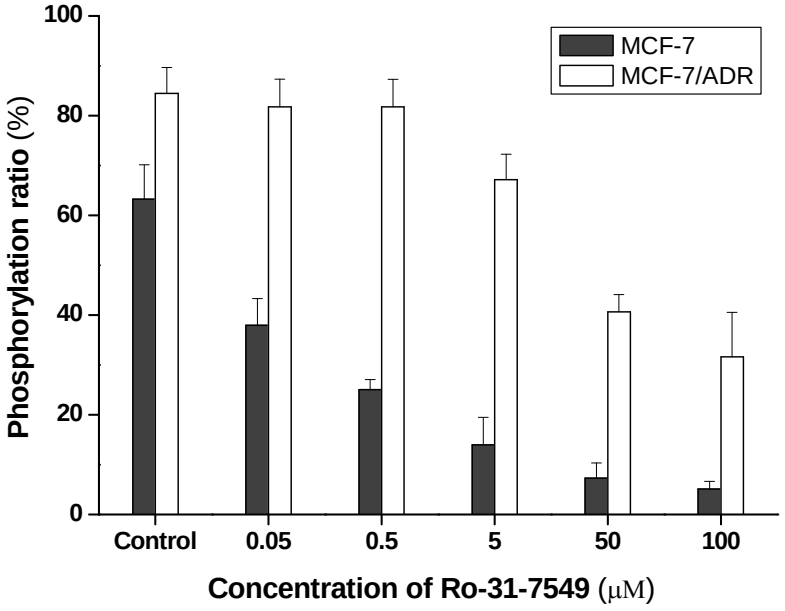


Figure 4.6. Inhibition of PKCα activity by the addition of protein kinase inhibitor (Ro-31-7549). After 6 h incubation with Ro-31-7549, cells were scrapped and sonicated at 4 °C. The samples was then centrifuged and the supernatant was used as cell lysates. The phosphorylation of the PKCα-specific peptide substrate with cell lysates at a 200 μg/mL protein concentration was performed at 37 °C for 1 h and analyzed by MALDI-TOF-MS. (n=3)

4.2.3 Doxorubicin-responsive alteration of PKCα activity and its inhibition by PKC inhibitor

To investigate the alteration of PKCα activity in the presence of doxorubicin, phosphorylation assay was performed in both MCF-7 and MCF-7/ADR cells after exposure to the different concentration of doxorubicin for a short period (6 h), in which the cell death in both cell lines was negligible. As shown in **Figure 4.7**, in MCF-7/ADR cells, the phosphorylation ratios of the PKCα-specific peptide substrate were gradually increased with increasing doxorubicin concentration. In contrast, there was no change in phosphorylation ratio in MCF-7 cells by doxorubicin treatment. Thus, MCF-7/ADR cells responds sensitively to doxorubicin through activation of PKCα.

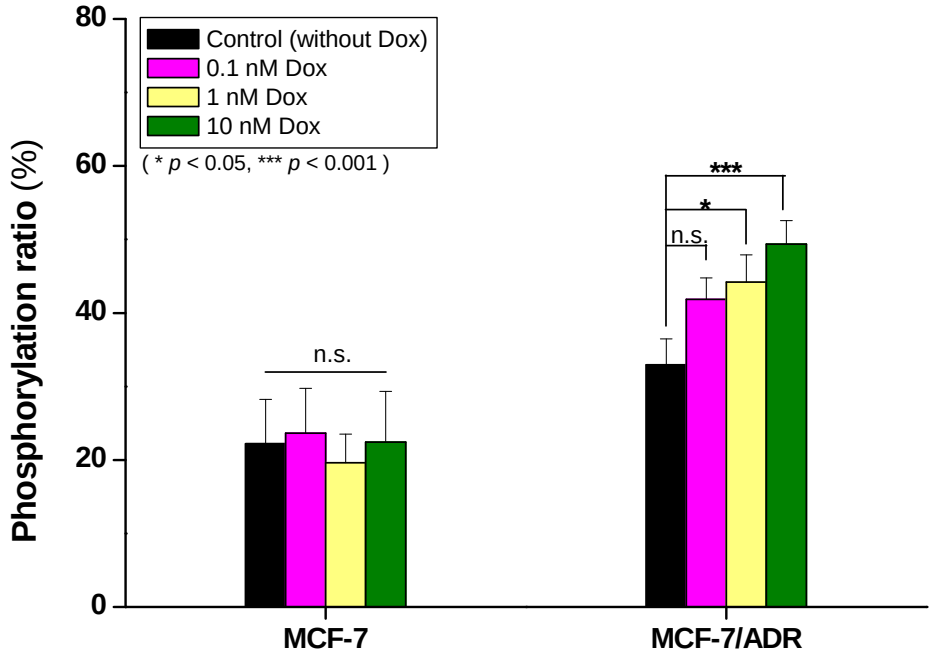


Figure 4.7. Effect of doxorubicin on phosphorylation ratio of the PKCα-specific peptide substrate with the different concentration of doxorubicin in both MCF-7 and MCF-7/ADR cells, respectively. After 6 h incubation with doxorubicin, cells were scrapped and sonicated at 4 °C. The samples were then centrifuged and the supernatant was used as cell lysates. The phosphorylation of the PKCα-specific peptide substrate with cell lysates at a 100 μg/mL protein concentration was performed at 37 °C for 1 h and analyzed by MALDI-TOF-MS. (n=3)

To assess the ability of Ro-31-7549 to modulate the levels of PKCα activity in the presence of doxorubicin, 50 μM Ro-31-7549 was used to compare the levels in MCF-7 and MCF-7/ADR cells. As expected, Ro-31-7549 induced the significant inhibition efficacy of phosphorylation reactions in both cells, irrespective of the presence of doxorubicin as shown in **Figure 4.8**.

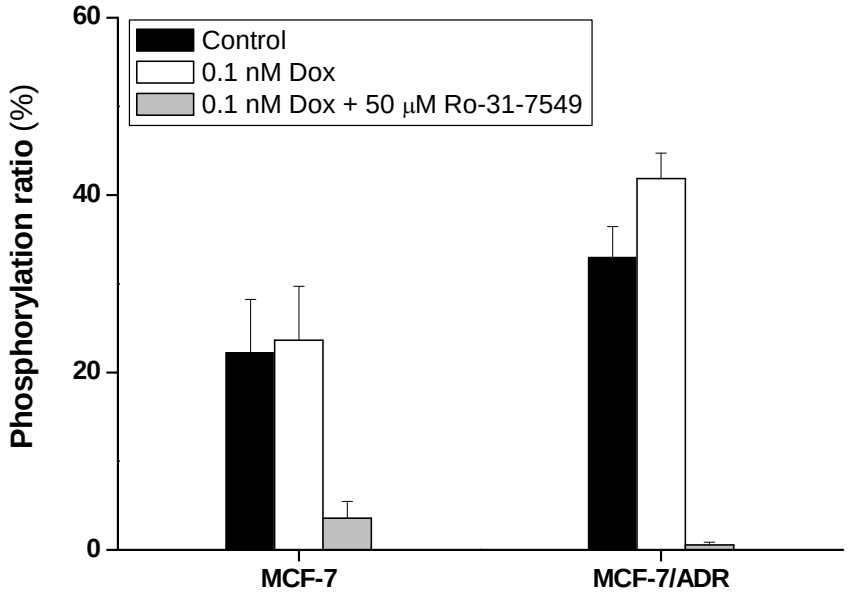


Figure 4.8. Phosphorylation ratio of the PKCα-specific peptide substrate with doxorubicin and Ro-31-7549 in MCF-7 and MCF-7/ADR cells. After 6 h incubation with both 0.1 nM doxorubicin and 50 μM Ro-31-7549, cells were scrapped and sonicated at 4 °C. The samples were then centrifuged and the supernatant was used as cell lysates. The phosphorylation of the PKCα-specific peptide substrate with cell lysates at a 100 μg/mL protein concentration was performed at 37 °C for 1 h and analyzed by MALDI-TOF-MS. (n=3)

4.2.4 Reversal of the resistance to doxorubicin by PKC inhibitor

Cellular accumulation of doxorubicin and its reversal were monitored by confocal microscopy in MCF-7 and MCF-7/ADR cells. As shown in **Figure 4.9**, in the absence of Ro-31-7549, a significant doxorubicin accumulation was observed in drug sensitive MCF-7 cells, whereas, their accumulation was substantially suppressed in drug resistant MCF-7/ADR cells. This lower accumulation of doxorubicin in MCF-7/ADR

cells was clearly distinct from that in MCF-7 cells, and showed increased efflux of doxorubicin by the overexpression of Pgp in resistance cells. In contrast, in the presence of Ro-31-7549, the cellular uptake of doxorubicin was extensively enhanced in MCF-7/ADR cells, indicating the significant suppression of Pgp-mediated doxorubicin efflux caused by the inhibition of PKCα activity by Ro-31-7549.

To further investigate its reversal via Ro-31-7549 in MCF-7/ADR, the cellular uptake of doxorubicin was monitored by a Tali^{TM} Image-Based Cytometer. As shown in **Figure 4.10**, the uptake of doxorubicin was not observed in the absent of Ro-31-7549, irrespective of the concentration of doxorubicin, whereas, their uptake was increased by the treatment of Ro-31-7549. These results indicated that the coadministration of PKC inhibitor (Ro-31-7549) improved the cellular uptake of doxorubicin probably because of the suppression of drug efflux in drug-resistant cells.

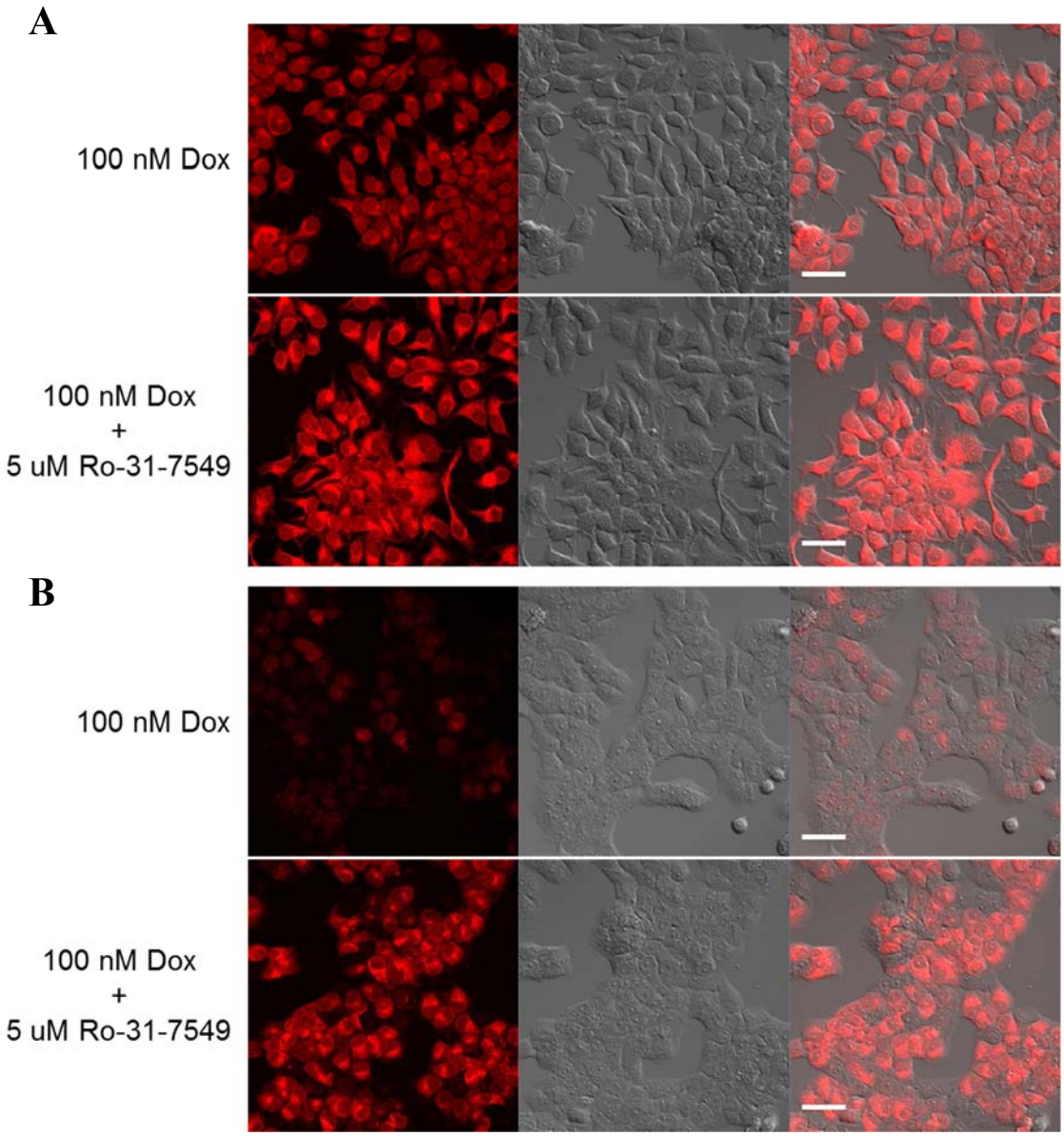


Figure 4.9. Intracellular distribution of doxorubicin in (A) MCF-7 and (B) MCF-7/ADR cells. Cellular accumulation and distribution of doxorubicin (100 nM) was observed by confocal microscopy. After treatment of doxorubicin for 2 h with/without pre-incubation of 5 μM Ro-31-7549 for 30 min, confocal images were obtained. The scale bar represents 50 μm.

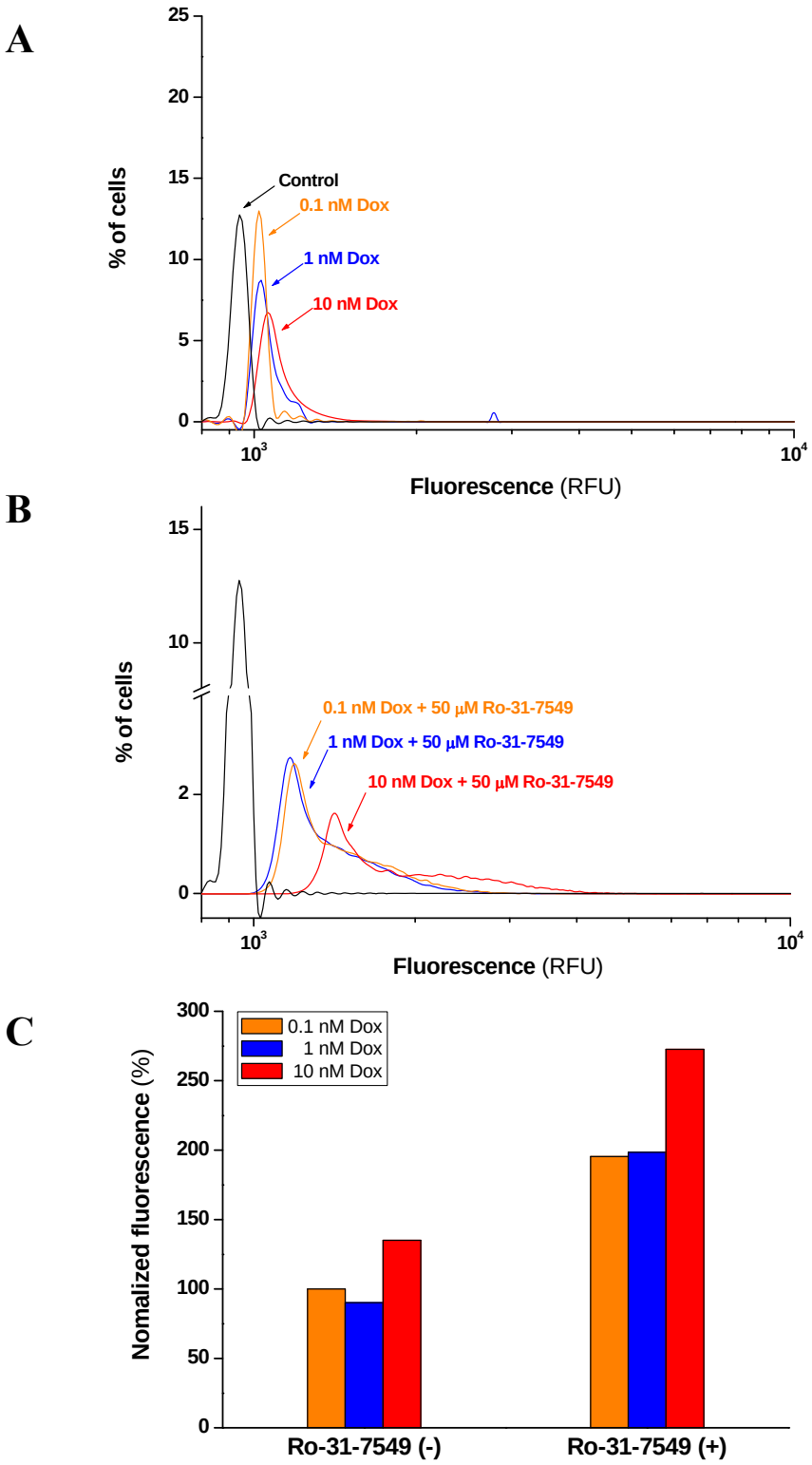


Figure 4.10. The effect of Ro-31-7549 on the accumulation of (A) doxorubicin alone and (B) both doxorubicin and Ro-31-7549 in resistant MCF-7/ADR cells. (C) The median of cellular doxorubicin fluorescence is normalized to cellular doxorubicin fluorescence in doxorubicin (0.1 nM) alone as control.

4.3 Summary

I demonstrated the enhanced doxorubicin accumulation in MCF-7/ADR cells, which is caused by the inhibition of PKCα activity. The effective regulation of PKCα activity could lead to a decrease of phosphorylation reactions by PKC inhibitor (Ro-31-7549), and consequently induce increased drug accumulation in drug-resistant MCF-7/ADR cells. This may suggest that the overexpression of Pgp in MDR cancer cells have been linked to PKCα activities, inducing phosphorylation reactions in the linker region of Pgp, and provide evidence of their regulatory mechanism. Although it still needs further investigation, the regulation of PKCα by Ro-31-7549 in this study represents the potential as an alternative modulator and can be expanded to coadministration with many other therapeutic anticancer drugs in MDR cancer cells.

4.4 Experimental section

Cell culture

MCF-7 cells were cultured in MEM containing 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 μg/mL insulin. MCF-7/ADR cells were a gift from Dr. Ick Chan Kwon (Center for Theragnosis at Korea Institute of Science and Technology, Seoul, Korea), and cultured in RPMI 1640 medium. All medium contained 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Gibco Invitrogen Co., Grand Island, NY, USA). The cell was harvested in a humidified atmosphere containing 5 % CO_2 and 95 % air at 37 °C.

Cell cytotoxicity of doxorubicin and PKC inhibitor (Ro-31-7549)

2×10^3 cells were seeded into a 96-well plate and cultured for 24 h at 37 °C in a 5 % CO_2 incubator. The medium in each well was replaced with 100 μL of fresh medium containing a different concentration of doxorubicin hydrochloride (0 to 100 nM) (Wako Pure Chemicals, Osaka, Japan) and PKC inhibitor (Ro-31-7549) (0 to 50 μM) (Merck Millipore, Billerica, MA, USA). After incubation for 24 or 72 h, the medium was replaced with 100 μL of fresh medium and then 10 μL of 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-8) (Dojindo Laboratories, Kumamoto, Japan) was added to the each well. The cells were incubated for a further 2 h. The cell viability (%) was calculated by the absorbance of the treated cells to that of the untreated control cells at 440 nm. This assay was performed in triplicate.

Phosphorylation assay of the PKCα-responsive peptide substrate

MCF-7 and MCF-7/ADR cells were plated at a density of 1×10^6 in 100-mm dish or 2×10^5 in 6-well plates at 37 °C in 2 mL medium containing 10 % FBS. At approximately 70-80 % confluency, cells were stimulated at a different concentration of

doxorubicin (0.01 to 10 nM) and Ro-31-7549 (0.05 to 100 μM) for 6 h, and the scrapped in 1 mL buffer [10 mM HEPES, 10 % sucrose, Complete™ protease inhibitor cocktail (EDTA-Free) (Roche, Basel, Switzerland)]. The samples were centrifuged at 5000 × g at 4 °C for 10 min and the supernatant was removed. A 0.1 mL of buffer was added in the precipitate and then sonicated twice at cooled condition for 15 sec. The samples were centrifuged again at 5000 × g at 4 °C for 15 min, and the resulting supernatant was used for the phosphorylation of the peptide substrate. The total protein concentration of cell lysates was measured by using the Bio-Rad Protein Assay Dye reagent (BIO-RAD Laboratories) with bovine serum albumin as the standard. The phosphorylation of the peptide substrate responding to PKCα in both MCF-7 and MCF-7/ADR cell lines was carried out in 100 μL buffer [10 mM HEPES, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.0 μg/mL diacylglycerol (DAG), 2.5 μg/mL phosphatidylserine (PS), and 200 μM ATP] containing 30 μM peptide substrate and cell lysates at a 100 or 200 μg/mL protein concentration. The phosphorylation was allowed to proceed for 1 h at 37 °C and then the resulting solutions were analyzed by MALDI-TOF-MS. The IC_{50} values were obtained from the GraphPad Prism Software.

Confocal laser scanning microscope (CLSM)

MCF-7 and MCF-7/ADR cells were plated at a density of 1×10^5 in 35-mm glass bottom dishes (Matsunami, Osaka, Japan) at 37 °C in 1 mL medium containing 10 % FBS for 24 h. The dishes were washed with PBS, and incubated with or without 5 μM Ro-31-7549 for 30 min, and the cells were further incubated with 100 μM doxorubicin for 2 h. The intracellular localization and accumulation of doxorubicin were observed by confocal microscopy (ZEISS LSM 700, Carl Zeiss, Oberlochen, Germarny) equipped with an EC Plan-Neofluar 20x/0.50 M27 objective. The images were obtained by using manufacturer-specified laser excitation wavelengths and emission filter sets and processed with Zeiss Zen 2010 software at excitation wavelengths of 488 nm for doxorubicin.

Cellular accumulation of doxorubicin

MCF-7/ADR cells were plated at a density of 5×10^4 in 24-well plates at 37 °C in medium containing 10 % FBS for 24 h. The medium in each well was washed with PBS and replaced with Opti-MEM (Gibco), and then incubated with or without 50 μM Ro-31-7549 in the different concentration of doxorubicin, 0, 0.1, 1 or 10 nM for 6 h. Estimation of cellular uptake was conducted by a Tali™ Image-Based Cytometer (Invitrogen).

4.5 References

(1) Ford, J. M.; Hait, W. N. *Pharmacol Rev* **1990**, *42*, 155.

(2) Ouar, Z.; Lacave, R.; Bens, M.; Vandewalle, A. *Cell Biol Toxicol* **1999**, *15*, 91.

(3) Zgurskaya, H. I.; Nikaido, H. *Mol Microbiol* **2000**, *37*, 219.

(4) Gottesman, M. M.; Fojo, T.; Bates, S. E. *Nat Rev Cancer* **2002**, *2*, 48.

(5) Thomas, H.; Coley, H. M. *Cancer Control* **2003**, *10*, 159.

(6) Jones, P. M.; George, A. M. *Cell Mol Life Sci* **2004**, *61*, 682.

(7) Deeley, R. G.; Westlake, C.; Cole, S. P. C. *Physiol Rev* **2006**, *86*, 849.

(8) Sarkadi, B. *J Physiol-London* **2001**, *535*, 3S.

(9) Sharom, F. J. *Pharmacogenomics* **2008**, *9*, 105.

(10) Aller, S. G.; Yu, J.; Ward, A.; Weng, Y.; Chittaboina, S.; Zhuo, R. P.; Harrell, P. M.; Trinh, Y. T.; Zhang, Q. H.; Urbatsch, I. L.; Chang, G. *Science* **2009**, *323*, 1718.

(11) Rees, D. C.; Johnson, E.; Lewinson, O. *Nat Rev Mol Cell Bio* **2009**, *10*, 218.

(12) Gradilone, A.; Raimondi, C.; Naso, G.; Silvestri, I.; Repetto, L.; Palazzo, A.; Gianni, W.; Frati, L.; Cortesi, E.; Gazzaniga, P. *Am J Clin Oncol-Canc* **2011**, *34*, 625.

(13) Fletcher, J. I.; Haber, M.; Henderson, M. J.; Norris, M. D. *Nat Rev Cancer* **2010**, *10*, 147.

(14) Krishna, R.; Mayer, L. D. *Eur J Pharm Sci* **2000**, *11*, 265.

(15) Tsuruo, T.; Iida, H.; Tsukagoshi, S.; Sakurai, Y. *Cancer Res* **1981**, *41*, 1967.

(16) Twentyman, P. R.; Fox, N. E.; White, D. J. *Br J Cancer* **1987**, *56*, 55.

(17) List, A. F.; Spier, C.; Greer, J.; Wolff, S.; Hutter, J.; Dorr, R.; Salmon, S.; Futscher, B.; Baier, M.; Dalton, W. *J Clin Oncol* **1993**, *11*, 1652.

(18) Spoelstra, E. C.; Westerhoff, H. V.; Pinedo, H. M.; Dekker, H.; Lankelma, J. *Eur J Biochem* **1994**, *221*, 363.

(19) Lavie, Y.; Cao, H. T.; Volner, A.; Lucci, A.; Han, T. Y.; Geffen, V.; Giuliano, A. E.; Cabot, M. C. *J Biol Chem* **1997**, *272*, 1682.

(20) Aszalos, A.; Thompson, K.; Yin, J. J.; Ross, D. D. *Anticancer Res* **1999**, *19*, 1053.

(21) Bogush, T.; Koldaeva, E.; Bogush, E.; Kushlinsky, N.; Gerstain, E. *Eur J Cancer* **2002**, *38*, S116.

(22) Qadir, M.; O'Loughlin, K. L.; Fricke, S. M.; Williamson, N. A.; Greco, W. R.; Minderman, H.; Baer, M. R. *Clin Cancer Res* **2005**, *11*, 2320.

(23) Boer, R.; Haas, S.; Schodl, A. *Eur J Cancer* **1994**, *30A*, 1117.

(24) Wilson, W. H.; Jamisdow, C.; Bryant, G.; Balis, F. M.; Klecker, R. W.; Bates, S. E.; Chabner, B. A.; Steinberg, S. M.; Kohler, D. R.; Wittes, R. E. *J Clin Oncol* **1995**, *13*, 1985.

(25) Wilson, W. H.; Bates, S. E.; Fojo, A.; Bryant, G.; Zhan, Z.; Regis, J.; Wittes, R. E.; Jaffe, E. S.; Steinberg, S. M.; Herdt, J.; Chabner, B. A. *J Clin Oncol* **1995**, *13*, 1995.

(26) Fisher, G. A.; Lum, B. L.; Hausdorff, J.; Sikic, B. I. *Eur J Cancer* **1996**, *32A*, 1082.

(27) Patterson, K. K.; Beckman, B. S.; Klotz, D. M.; Mallia, C. M.; Jeter, J. R. *J Cancer Res Clin* **1996**, *122*, 465.

(28) Tidefelt, U.; Liliemark, J.; Gruber, A.; Liliemark, E.; Sundman-Engberg, B.; Juliusson, G.; Stenke, L.; Elmhorn-Rosenborg, A.; Mollgard, L.; Lehman, S.; Xu, D.; Covelli, A.; Gustavsson, B.; Paul, C. *J Clin Oncol* **2000**, *18*, 1837.

(29) Peck, R. A.; Hewett, J.; Harding, M. W.; Wang, Y. M.; Chaturvedi, P. R.; Bhatnagar, A.; Ziessman, H.; Atkins, F.; Hawkins, M. J. *J Clin Oncol* **2001**, *19*, 3130.

(30) Bramwell, V. H.; Morris, D.; Ernst, D. S.; Hings, I.; Blackstein, M.; Venner, P. M.; Ette, E. I.; Harding, M. W.; Waxman, A.; Demetri, G. D. *Clin Cancer Res* **2002**, *8*, 383.

(31) Toppmeyer, D.; Seidman, A. D.; Pollak, M.; Russell, C.; Tkaczuk, K.; Verma, S.; Overmoyer, B.; Garg, V.; Ette, E.; Harding, M. W.; Demetri, G. D. *Clin Cancer Res* **2002**, *8*, 670.

(32) Borchers, C.; Boer, R.; Klemm, K.; Figala, V.; Denzinger, T.; Ulrich, W. R.; Haas, S.; Ise, W.; Gekeler, V.; Przybylski, M. *Mol Pharmacol* **2002**, *61*, 1366.

(33) Evers, R.; Kool, M.; Smith, A. J.; van Deemter, L.; de Haas, M.; Borst, P. *Brit J Cancer* **2000**, *83*, 366.

(34) Rubin, E. H.; de Alwis, D. P.; Pouliquen, I.; Green, L.; Marder, P.; Lin, Y.; Musanti, R.; Grospe, S. L.; Smith, S. L.; Toppmeyer, D. L.; Much, J.; Kane, M.; Chaudhary, A.; Jordan, C.; Burgess, M.; Slapak, C. A. *Clin Cancer Res* **2002**, *8*, 3710.

(35) Rubin, E. H.; de Alwis, D. P.; Pouliquen, I.; Green, L.; Marder, P.; Lin, Y.; Musanti, R.; Grospe, S. L.; Smith, S. L.; Toppmeyer, D. L.; Much, J.; Kane, M.; Chaudhary, A.; Jordan, C.; Burgess, M.; Slapak, C. A. *Clin Cancer Res* **2002**, *8*, 3710.

(36) Agrawal, M.; Abraham, J.; Balis, F. M.; Edgerly, M.; Stein, W. D.; Bates, S.; Fojo, T.; Chen, C. C. *Clin Cancer Res* **2003**, *9*, 650.

(37) Fracasso, P. M.; Goldstein, L. J.; de Alwis, D. P.; Rader, J. S.; Arquette, M. A.; Goodner, S. A.; Wright, L. P.; Fears, C. L.; Gazak, R. J.; Andre, V. A.; Burgess, M. F.; Slapak, C. A.; Schellens, J. H. *Clin Cancer Res* **2004**, *10*, 7220.

(38) Pusztai, L.; Wagner, P.; Ibrahim, N.; Rivera, E.; Theriault, R.; Booser, D.; Symmans, F. W.; Wong, F.; Blumenschein, G.; Fleming, D. R.; Rouzier, R.; Boniface, G.; Hortobagyi, G. N. *Cancer* **2005**, *104*, 682.

(39) Fox, E.; Bates, S. E. *Expert Rev Anticanc* **2007**, *7*, 447.

(40) Abraham, J.; Edgerly, M.; Wilson, R.; Chen, C.; Rutt, A.; Bakke, S.; Robey, R.; Dwyer, A.; Goldspiel, B.; Balis, F.; Van Tellingen, O.; Bates, S. E.; Fojo, T. *Clin Cancer Res* **2009**, *15*, 3574.

(41) Kuhnle, M.; Egger, M.; Muller, C.; Mahringer, A.; Bernhardt, G.; Fricker, G.; Konig, B.; Buschauer, A. *J Med Chem* **2009**, *52*, 1190.

(42) Kelly, R. J.; Draper, D.; Chen, C. C.; Robey, R. W.; Figg, W. D.; Piekarz, R. L.; Chen, X. H.; Gardner, E. R.; Balis, F. M.; Venkatesan, A. M.; Steinberg, S. M.; Fojo, T.; Bates, S. E. *Clin Cancer Res* **2011**, *17*, 569.

(43) Loo, T. W.; Clarke, D. M. *J Biol Chem* **2001**, *276*, 14972.

(44) Allen, J. D.; van Loevezijn, A.; Lakhai, J. M.; van der Valk, M.; van Tellingen, O.; Reid, G.; Schellens, J. H. M.; Koomen, G. J.; Schinkel, A. H. *Mol Cancer Ther* **2002**, *1*, 417.

(45) Doyle, L. A.; Ross, D. D. *Oncogene* **2003**, *22*, 7340.

(46) Mazel, M.; Clair, P.; Rousselle, C.; Vidal, P.; Scherrmann, J. M.; Mathieu, D.; Temsamani, J. *Anti-Cancer Drug* **2001**, *12*, 107.

(47) Dubikovskaya, E. A.; Thorne, S. H.; Pillow, T. H.; Contag, C. H.; Wender, P. A. *P Natl Acad Sci USA* **2008**, *105*, 12128.

(48) Abu Ajaj, K.; Graeser, R.; Kratz, F. *Breast Cancer Res Tr* **2012**, *134*, 117.

(49) Pearson, J. W.; Fogler, W. E.; Volker, K.; Usui, N.; Goldenberg, S. K.; Gruys, E.; Riggs, C. W.; Komschlies, K.; Wiltrout, R. H.; Tsuruo, T.; Pastan, I.; Gottesman, M. M.; Longo, D. L. *J Natl Cancer I* **1991**, *83*, 1386.

(50) Nishioka, Y.; Yano, S.; Fujiki, F.; Mukaida, N.; Matsushima, K.; Tsuruo, T.; Sone, S. *Int J Cancer* **1997**, *71*, 170.

(51) Goda, K.; Fenyvesi, F.; Bacso, Z.; Nagy, H.; Marian, T.; Megyeri, A.; Krasznai, Z.; Juhasz, I.; Vecsernyes, M.; Szabo, G. *J Pharmacol Exp Ther* **2007**, *320*, 81.

(52) Matsuo, H.; Wakasugi, M.; Takanaga, H.; Ohtani, H.; Naito, M.; Tsuruo, T.; Sawada, Y. *J Control Release* **2001**, *77*, 77.

(53) Shaik, M. S.; Chatterjee, A.; Singh, M. *J Pharm Pharmacol* **2004**, *56*, 899.

(54) Patel, N. R.; Rathi, A.; Mongayt, D.; Torchilin, V. P. *Int J Pharmaceut* **2011**, *416*, 296.

(55) van Vlerken, L. E.; Duan, Z. F.; Seiden, M. V.; Amiji, M. M. *Cancer Res* **2007**, *67*, 4843.

(56) Yadav, S.; van Vlerken, L. E.; Little, S. R.; Amiji, M. M. *Cancer Chemoth Pharm* **2009**, *63*, 711.

(57) Stuart, M. A. C.; Huck, W. T. S.; Genzer, J.; Muller, M.; Ober, C.; Stamm, M.; Sukhorukov, G. B.; Szleifer, I.; Tsukruk, V. V.; Urban, M.; Winnik, F.; Zauscher, S.; Luzinov, I.; Minko, S. *Nat Mater* **2010**, *9*, 101.

(58) Wang, Y. C.; Wang, F.; Sun, T. M.; Wang, J. *Bioconjugate Chem* **2011**, *22*, 1939.

(59) Lee, E. S.; Gao, Z. G.; Kim, D.; Park, K.; Kwon, I. C.; Bae, Y. H. *J Control Release* **2008**, *129*, 228.

(60) Kim, D.; Lee, E. S.; Oh, K. T.; Gao, Z. G.; Bae, Y. H. *Small* **2008**, *4*, 2043.

(61) Li, X. R.; Li, P. Z.; Zhang, Y. H.; Zhou, Y. X.; Chen, X. W.; Huang, Y. Q.; Liu, Y. *Pharm Res-Dordr* **2010**, *27*, 1498.

(62) Shen, J. N.; He, Q. J.; Gao, Y.; Shi, J. L.; Li, Y. P. *Nanoscale* **2011**, *3*, 4314.

(63) Batrakova, E. V. *Nanomedicine (Lond)* **2011**, *6*, 1492.

(64) Li, L. L.; Huang, X. L.; Liu, T. L.; Liu, H. Y.; Hao, N. J.; Chen, D.; Zhang, Y. Q.; Li, L. F.; Tang, F. Q. *J Nanosci Nanotechno* **2012**, *12*, 4458.

(65) Pan, L. M.; Liu, J. A.; He, Q. J.; Wang, L. J.; Shi, J. L. *Biomaterials* **2013**, *34*, 2719.

(66) Wong, H. L.; Rauth, A. M.; Bendayan, R.; Manias, J. L.; Ramaswamy, M.; Liu, Z. S.; Erhan, S. Z.; Wu, X. Y. *Pharm Res-Dordr* **2006**, *23*, 1574.

(67) Sun, Y.; Chen, X. Y.; Zhu, Y. J.; Liu, P. F.; Zhu, M. J.; Duan, Y. R. *J Mater Chem* **2012**, *22*, 5128.

(68) Zhang, P.; Ling, G. X.; Pan, X. L.; Sun, J.; Zhang, T. H.; Pu, X. H.; Yin, S. L.; He, Z. G. *Nanomed-Nanotechnol* **2012**, *8*, 185.

(69) Gottesman, M. M. *Annu Rev Med* **2002**, *53*, 615.

(70) Malam, Y.; Loizidou, M.; Seifalian, A. M. *Trends Pharmacol Sci* **2009**, *30*, 592.

(71) Hu, C. M. J.; Zhang, L. F. *Curr Drug Metab* **2009**, *10*, 836.

(72) Chambers, T. C.; Mcavoy, E. M.; Jacobs, J. W.; Eilon, G. *J Biol Chem* **1990**, *265*, 7679.

(73) Budworth, J.; Gant, T. W.; Gescher, A. *Brit J Cancer* **1997**, *75*, 1330.

(74) Idriss, H.; Urquidi, V.; Basavappa, S. *Cancer Chemoth Pharm* **2000**, *46*, 287.

(75) Bates, S. E.; Lee, J. S.; Dickstein, B.; Spolyar, M.; Fojo, A. T. *Biochemistry-Us* **1993**, *32*, 9156.

(76) Chambers, T. C.; Pohl, J.; Glass, D. B.; Kuo, J. F. *Biochem J* **1994**, *299*, 309.

(77) Ahmad, S.; Safa, A. R.; Glazer, R. I. *Biochemistry-Us* **1994**, *33*, 10313.

(78) Fine, R. L.; Chambers, T. C.; Sachs, C. W. *Oncologist* **1996**, *1*, 261.

(79) Idriss, H. T.; Hannun, Y. A.; Boulpaep, E.; Basavappa, S. *J. Physiol* **2000**, *524*, 629.

(80) Choi, C. H. *Cancer Cell Int* **2005**, *5*.

(81) Scambia, G.; Ranelletti, F. O.; Panici, P. B.; Devincenzo, R.; Bonanno, G.; Ferrandina, G.; Piantelli, M.; Bussa, S.; Rumi, C.; Cianfriglia, M.; Mancuso, S. *Cancer Chemoth Pharm* **1994**, *34*, 459.

(82) Doyle, L. A.; Yang, W. D.; Abruzzo, L. V.; Krogmann, T.; Gao, Y. M.; Rishi, A. K.; Ross, D. D. *P Natl Acad Sci USA* **1998**, *95*, 15665.

(83) Ross, D. D.; Yang, W. D.; Abruzzo, L. V.; Dalton, W. S.; Schneider, E.; Lage, H.; Dietel, M.; Greenberger, L.; Cole, S. P. C.; Doyle, L. A. *J Natl Cancer I* **1999**, *91*, 429.

(84) Wilkinson, S. E.; Parker, P. J.; Nixon, J. S. *Biochem J* **1993**, *294*, 335.

(85) Murphy, C. T.; Westwick, J. *Biochem J* **1992**, *283*, 159.

(86) Kang, J. H.; Asai, D.; Yamada, S.; Toita, R.; Oishi, J.; Mori, T.; Niidome, T.; Katayama, Y. *Proteomics* **2008**, *8*, 2006.

(87) Yu, G.; Ahmad, S.; Aquino, A.; Fairchild, C. R.; Trepel, J. B.; Ohno, S.; Suzuki, K.; Tsuruo, T.; Cowan, K. H.; Glazer, R. I. *Cancer Commun* **1991**, *3*, 181.

(88) Blobe, G. C.; Sachs, C. W.; Khan, W. A.; Fabbro, D.; Stabel, S.; Wetsel, W. C.; Obeid, L. M.; Fine, R. L.; Hannun, Y. A. *J Biol Chem* **1993**, *268*, 658.

CHAPTER 5 Conclusions

The availability of intracellular signal transduction and its abnormal activities in many cancers has potential in medical and pharmaceutical applications. The research described herein presents great opportunities for efficient cancer-specific gene delivery, and one potential for overcoming drug resistance in cancer chemotherapy, focusing on abnormally activated intracellular signaling molecules, especially protein kinase Cα, as an attractive target in many cancers.

In chapter 2, I presented detailed characterization of the LPEI-peptide conjugate as a possible candidate of cancer cell-specific gene carrier both *in vitro* and *in vivo*. The specific and efficient response to target PKCα was significantly improved in many cancers than our previous PPC polymer which used in polyacrylamide backbone. In addition, their intracellular trafficking exhibited the effective cellular uptake and capable of endosomal escape of polyplexes caused by a high pH buffering capacity of LPEI main chain at around 5 to 6, consistent with their efficient transgene expression both *in vitro* and *in vivo*. Thus, this cellular signal-responsive system may serve as a potential alternative for a highly disease cell-specific gene delivery.

In chapter 3, I proposed a new carrier to stabilize polyplexes through the additional hydrophobic interaction. The cellular uptake of polyplexes was enhanced via hydrophobic interaction with a simple modification of a long alkyl chain as a spacer between the LPEI main chain and a substrate peptide, presenting no effect of this modification on the reactivity of the substrate peptide toward target PKCα and the endosomal escaping ability. Because of these advantageous characteristics of the hydrophobically modified polyplexes, the polyplexes showed excellent performance in PKCα-responsive gene expression. Thus, the improved stability of the LPEI peptide conjugate provides a further perspective as a promising gene carrier.

In chapter 4, I proposed one potential for reversal of drug resistance by regulating the activity of PKCα as an appropriate modulator in cancer chemotherapy. The enhanced doxorubicin accumulation was observed in drug-resistance MCF-7/ADR cells caused by the inhibition of PKCα activities. The effective inhibition of PKCα activity might lead to a decrease of phosphorylation reactions in P-glycoprotein, which

is mainly implicated with drug efflux, and consequently induce increased drug accumulation in drug-resistant MCF-7/ADR cells. This may provide evidence that PKCα is related with drug efflux caused by Pgp phosphorylation and suggest that the regulation of PKCα has a potential for reversing drug resistance.

These novel approaches that I have descried in this thesis may provide an extension of current gene delivery system and successful cancer chemotherapy. Although there still remain many obstacles and further investigations, I hope that this thesis contributes to mark a step toward creating better systems in a broader range of medical and pharmaceutical research.