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研究課題名（和文）Exploiting Neomycin-RNA complexation to create PIC micelle based efficient transportation for therapeutic siRNA

研究課題名（英文）Exploiting Neomycin-RNA complexation to create PIC micelle based efficient transportation for therapeutic siRNA

研究代表者

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研究成果の概要（和文）：The purpose of the current research is to construct PolyIonComplex (PIC) where, a widely used amimoglycoside antibiotic Neomycin, that has specific RNA-binding ability will be used as polycationic segments to hold polyanionic siRNA or other nucleic acids for cancer therapy.

研究成果の概要（英文）：The aim of this research is to construct PolyIonComplex (PIC), where, a widely used amimoglycoside antibiotic Neomycin, that has specific RNA-binding ability and also has anti-angiogenin property, will be used as polycationic segments to hold polyanionic siRNA or other nucleic acids for cancer-therapy. To achieve this purpose we designed a novel polymer conjugated with azide functionalized neomycin through click chemistry between the azide function of neomycin and alkyne function of polymer. Alkyne function was introduced in poly(ethylene glycol)-b-poly(β-benzyl-aspartamide) polymer via aminolysis reaction utilizing propargyl amine. We utilized dual armed PEG with each arm having molecular weight of around 40 KD and with only 6 repeating units of poly amino acid segments. This polymer was designed to conjugate calculated no of Neomycin units so to construct PolyIonComplex with minimum molar ratio of polymer and thus ultimately achieve very small size (around 25 nm) PIC.

研究分野：　nano materials

キーワード：　Neomycin　PolyIionComplex　siRNA　polymer　Aminoglycoside

Background of the Research

RNA interference has been the most vital advancement in the field of cell-biology in decades with enormous therapeutic potential to manage a large array of diseases including malignancy. However, the effective systemic *in vivo* delivery of small interfering RNA (siRNA) to tumors remains a formidable challenge mainly due to their instability in biological fluids, and limited ability to reach the target site. Although there are significant progressions in siRNA design and modifications with an aim to increase stability and reduce immunogenicity [Lorenzer C. *et al*, Going beyond the liver: Progress and challenges of targeted delivery of siRNA therapeutics. *J. Control. Release*, 2015, 203 1-15], there is still a need for efficient cellular uptake and target site accumulation.

Polyion complex (PIC) micelles, prepared through electrostatic interaction between PEG-block-polycation copolymers and nucleic acids, demonstrate remarkable properties as delivery vehicles for plasmid DNA or oligo-DNA. With the core-shell structure having the core surrounded by a dense PEG corona, these PIC micelles offer excellent colloidal stability in extracellular media, protection of incorporated DNA against enzymatic degradation, and prolonged blood circulation [Harada-shiba *et al.* Polyion complex micelles as vectors in gene therapy-pharmacokinetics and in vivo gene transfer. *Gene Ther*. 2002, **9**, 407]. However, it is known that siRNA is more difficult to incorporate in PIC micelle structures than DNA because of the short and rigid nature of siRNA that often results in larger and more loosely packed particles compared to DNA after complexing with carrier polycations. Therefore, structural alteration of the polycations used for complexation might improve micelle stability for delivery of siRNA [Kataoka et al, Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. *Biomacromolecules*. 2009; **12**, 119]. Alternatively, using polycationic segments having inherent specific-binding ability with RNA molecules might prove advantageous to effectively complex and deliver siRNA to the target site. Aminoglycosides (AGs) are natural compounds already widely used as antibiotics that provide a versatile polycationic framework. Neomycin B, a broad-spectrum AG antibiotic, is effective against both gram-negative and gram-positive organisms and exerts its antibacterial properties by binding to particular sites on RNA molecules in the bacterial ribosome. Neomycin B also binds to a variety of viral mRNAs such as HIV-1 RRE and TAR. The binding of neomycin B to these target RNAs is mediated mainly through electrostatic and hydrogen bonding interaction between the negatively charged phosphate backbone of the RNA and positively charged amino functional groups of neomycin B. Presence of six amino groups and also inherent conformational adaptability through the glycosidic connection in neomycin B, permits the optimum structural adjustment to effectively bind with diverse RNA targets. [Herman *et al*, Molecular Recognition of RNA by neomycin and a Restricted Neomycin Derivative, Angew. Chem. Int. Ed. 2005, 44, 5329 –5334]. Opportunistically, this auspicious RNA-neomycin B binding ability can be exploited to engineer a novel delivery platform for the efficient transport of RNA-based therapeutics to the target cells. Moreover, having six amino groups with different pKa values (ranging from 5.7 to 8.8) might also proof advantageous to reach the sophisticated balance between stability of PIC micelles in extracellular media and efficient release of siRNA to the cytoplasm of target cells. Multifunctional nature of neomycin B, affords selective structural modification [Quader *et al*., Multisite modification of neomycin B: combined Mitsunobu and click chemistry approach. *J Org Chem*. 2007; **72**:1962], allowing planned chemical conjugation to assemble neomycin B with base polymer (Scheme 1 and 2).

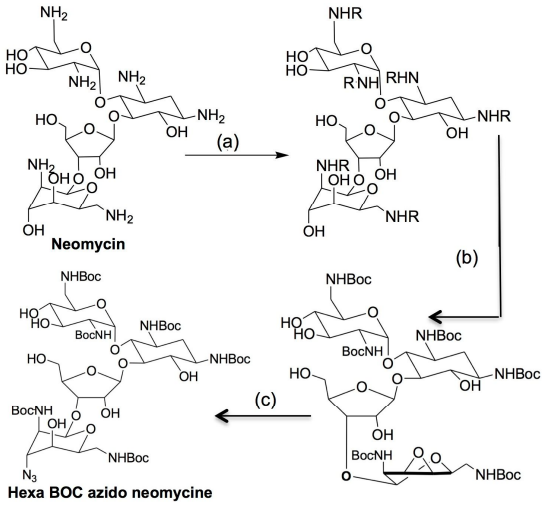
Purpose of the Research

The purpose of the current research project is to construct a multifunctional-delivery system using **PIC micelles**, where, an widely used **amimoglycoside** antibiotic **neomycin B**, that has specific RNA-binding ability and also has **anti-angiogenin** property, will be used as **polycationic** segments to accommodate polyanionic **siRNA** as a PolyIonComplex.

Construction of neomycin B based PIC micelle

1. **Functionalized neomycin B to allow viable conjugation with the base polymer**

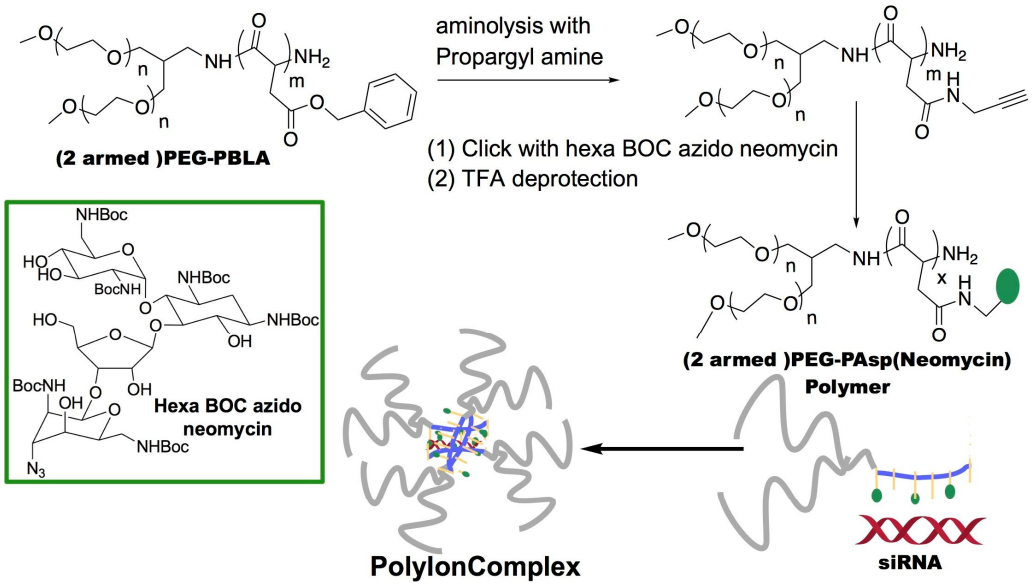
Azide functionalized neomycin was prepared according to the literature procedure [Quader *et al*., Multisite modification of neomycin B: combined Mitsunobu and click chemistry approach. *J Org Chem.* 2007; **72**:1962]. The reaction sequence for synthetic modification of neomycin B is highlighted in **Scheme 1 (a)** *tert*-butoxycarbamate (BOC) protecting group was introduced to block the 6 amine functional group; **(b)** performing Mitsunobu dehydration reaction on BOC protected neomycin B an epoxide-neomycin B derivative has been prepared and (c) then ring-opening reaction with azide furnished azido-neomycin derivative. This newly introduced azide function was then used to click with the base polymer (scheme 2).



Scheme 1

(a) $(Boc)_2O$, (10eq), Et_3N, $MeOH/H_2O$, 55^0C, overnight (b) Triphenylphosphine, diisopropyl azodicarboxylate, toluene, RT, overnight (c) NaN_3, rt, DMF, 2 days

Synthesis of 2 armed block copolymer, (2 armed) PEG-PBLA- A block copolymer, PEGasus-*block*- poly(β-benzyl L-aspartate) (2 armed PEGPBLA), was synthesized *via* ring opening polymerization of β-Benzyl L-aspartate N-carboxy anhydride initiated by PEGasus-NH_2 terminal primary amino group (M_W of PEG = 78k, degree of polymerization 6) following a previously reported procedure [M. Yokoyama, G.S. Kwon, T. Okano, Y. Sakurai, T. Seto, K. Kataoka, Preparation of micelle-forming polymer–drug conjugates, Bioconjug. Chem. 3 (1992) 295–301]. Alkyne functional group was introduced to the 2 armed-PEG-PBLA polymer by aminolysis reaction using propargylamine. Azido neomycin was then conjugated (3-4 units) to the polymer using copper catalyzed click reaction. Finally deprotection of the six amine-functions of conjugated neomycin B



Scheme 2

was done using trifluroaceticacid to facilitate the polycationic segment of the base polymer. PolyIonComplex with siRNA was prepared by mixing neomycin B conjugated base polymer and siRNA in aqueous solution. In this stage, different molar ratio of conjugated neomycin B and siRNA was tested to see which mixing ratio produces particles with optimal size and stability. The

polymer/siRNA ratio was expressed as the nitrogen/phosphate (N/P) ratio.

Physicochemical characterization of the prepared neomycin B based PIC micelle

Fluorescence correlation spectroscopy (FCS) analysis. Stable PolyIonComplex formation was confirmed by determining the diffusion time of the complex using Fluorescence correlation spectroscopy (FCS) analysis. For this experiment Cy-5 labeled siRNA was used to form the complex. The diffusion time was further converted to the diffusion coefficient (D_C) based on a reference of Cy5 dye. The hydrodynamic diameter (D_H) was calculated from the Stokes-Einstein equation: $D_H = k_B T/3\pi\eta D_C$, where k_B is the Boltzmann constant, T is the temperature, and η is the solvent viscosity. The correlation of size of the PolyIonComplex with different N/P ratio was shown in figure 1. The size of naked siRNA used in determined to be 6 nm. The size of the PolyIonComplex with N/P ratio 2 was found to be 9 nm which gradually increased with N/P ratio and ultimately plateaued at around 25 nm.

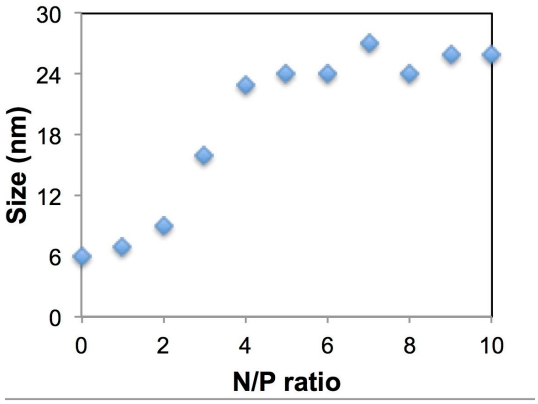


Figure 1- Size as hydrodynamic diameter (nm) of PolyIonComplex prepared from Polymer bound neomycin and Cy5-labeled siRNA at increasing N/P ratios.

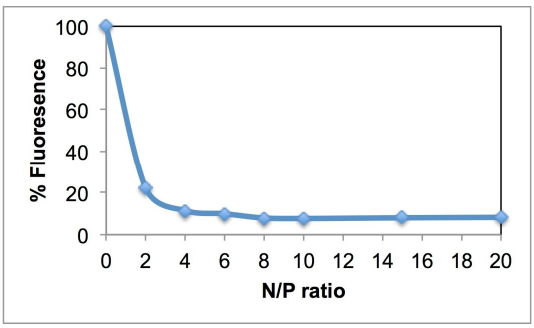


Figure 2-Relative binding affinity measured by ethidium bromide displacement assay of Polymer-Neomycin polycatiomer with siRNA at varying N/P ratios.

Ethidium bromide exclusion assay—The binding of polymer bound neomycin to siRNA was examined by the fluorescence quenching method based on ethidium bromide (EtBr). Stable complex formation can be observed (**Figure 2**) when N/P ratio was 2 and this binding remained constant with higher N/P ratio.

Evaluation of biological properties, including *in vitro* and *in vivo*-

Instability in the blood and poor cellular uptake following systemic administration are two of the major limitations for siRNA-based therapy.

Cellular Uptake assay- Cellular delivery of siRNA (Cy-5 labeled) by neomycin B conjugated PolyIonComplex was done in MDAMB231 cells cultured as monolayers. Cells were incubated for 24 hours with PolyIonComplex of different N/P ratio and washed with fresh media and labeled the nucleus with hoechst before taking image by confocal laser scanning microscopy. As shown in figure 3, complex prepared from different N/P ratio (2, 6 and 10), all are taken up by cells.

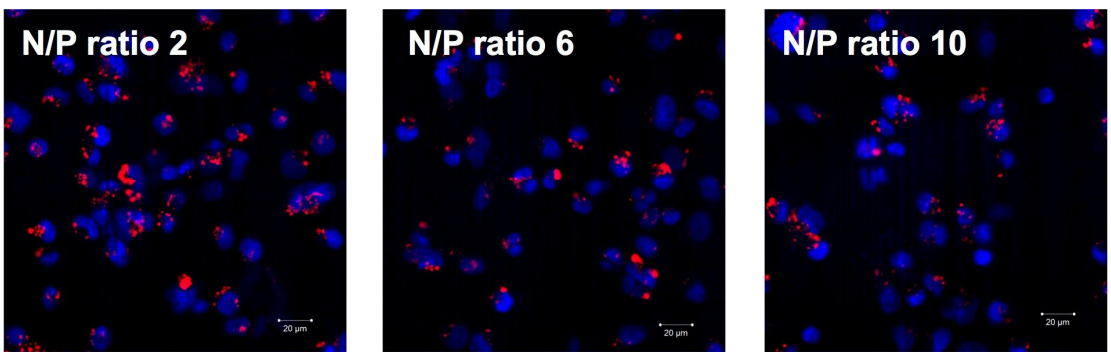


Figure 3- Cellular uptake assay in MDAMB231 mono-layered cells of PolyIonComplex with different N/P ratio. Blue- Hoechst, Red- Cy5 labeled siRNA.

Blood circulation profile of PolyIonComplex-

Prolonged blood circulation profile of **PolyIonComplex** was examined by intravital confocal micro-videography technique (Nikon A1R). Immediately after intravenous injection Cy5-siRNA complexed with polymer bound neomycin was observed flowing inside the blood vessels within the earlobe area of the experimental mouse and remained in the blood circulation even after 1.5 hours (N/P ratio 10) as shown in figure 4. On the other hand naked siRNA was washed out from the blood stream within 30 minutes of IV injection. The fluorescence intensity of Cy 5 in the bloodstream was normalized to the observed maximum intensity.

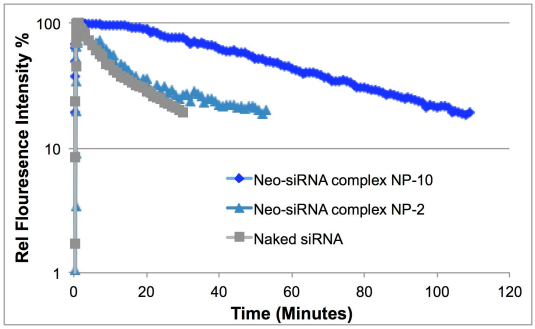


Figure 4- Blood circulation profile of **PolyIonComplex** or naked siRNA.

Conclusion- In conclusion, a novel neomycin bound block copolymer was designed and synthesized that could form stable PolyIonComplex with siRNA. We utilized dual armed PEG with each arm having molecular weight of around 39 KD and with only 6 repeating units of poly amino acid segments. This unique polymer was designed to conjugate 3-4 units of Neomycin so to construct PolyIonComplex with minimum molar ratio of polymer and thus ultimately achieve very small size (around 25 nm) PolyIonComplex. These PICs could effectively delivery siRNA inside the cell and also show prolonged blood circulation property.

Main Publication generated utilizing the research funding-

1. **Sabina Quader** and Kazunori Kataoka. Nanomaterial-Enabled Cancer Therapy. Molecular Therapy. 2017, 25, 1501-1513. Q1; IF=6.68
2. **Sabina Quader**, Xueying Liu, i-Chun Chen, Peng Mi, Tsukasa Chida, Takehiko Ishii, Yutaka Miura, Nobuhiro Nishiyama, Horacio Cabral and Kazunori Kataoka. cRGD peptide-installed epirubicin-loaded polymeric micelles for effective targeted therapy against brain tumors. J. Control. Release. 2017, 258, 56-66. Q1; IF=7.78
3. Juanjuan Zhang, Hiroaki Kinoh (2nd of 9), **Sabina Quader** (5th of 9), Kazunori Kataoka, Effective treatment of drug resistant recurrent breast tumors harboring cancer stem-like cells by staurosporine/epirubicin co-loaded polymeric micelles. J. Control. Release 264 (2017) 127-135. Q1; IF=7.78
4. Hailiang Wu**, Sabina Quader** (4th of 12 authors), Kazunori Kataoka, Proteasome Inhibitor-Loaded Micelles Enhance Antitumor Activity Through Macrophage Reprogramming by NF-κB Inhibition. J Pharm Sci. 106 (2017) 2438-2446. Q1; IF=2.7

Conference Presentation

Invited Lecturer

1. **Sabina Quader** and Kazunori Kataoka. "Targeting cancer using stimuli-sensitive polymeric micelle." March, 2018. International Symposium on Biorelated Polymers: 255th ACS National Meeting. New Orleans, USA (45 minutes).
2. **Sabina Quader**. "Nanocarrier system for cancer therapy." July, 2017. Summer School, Nanosciences, Fundamental and Applications Emphasizing in Nanomedicine. Institute of Technology Bandung, Indonesia. (4 lectures, totaling 6 hours).

Oral Presentation

1. **Sabina Quader**, Xueying Liu, Hiraoki Kinoh, Kazunori Kataoka. Tuning the Drug Release Kinetics of pH-responsive Polymer Micelle to achieve better therapy outcome in Glioblastoma model, 67th SPSJ Annual Meeting. May 2018.
2. **Sabina Quader**, Xueying Liu, Horacio Cabral, Kazunori Kataoka, Safe and effective transport of anti-cancer drug, thru pH-sensitive polymeric micelle., 66th SPSJ Annual Meeting. May 2017.