様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Development of nanovesicles with antisense oligonucleotides-embedded membrane and encapsulated RNase H in the cavity for cooperative gene knockdown

研究課題名（英文）Development of nanovesicles with antisense oligonucleotides-embedded membrane and encapsulated RNase H in the cavity for cooperative gene knockdown

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交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：一連の化学修飾ヌクレオチドとグアニジニル化PEG-b-ポリペプチドを使用して小胞PICを作製し、RNaseHを含むヌクレオチドを培養細胞に安定して共生させました。100 nmサイズの小胞構造は、ヌクレオチドのポリイオンペアとPEG-P（Asp-AG / G8O）によって、生理学的環境で正常に安定化されました。安定化されたヌクレオチドが埋め込まれたナノベシクルは、物理的捕捉により、RNaseHを効果的にカプセル化しました。 ナノベシクルは、ヌクレオチドとRNase Hの培養癌細胞への効率的な共生を可能にし、細胞毒性を無視して大幅に強化された遺伝子ノックダウン効果をもたらしました。

研究成果の学術的意義や社会的意義
This strategy can provide a versatile platform that allows for enhanced cooperative functions between small nucleic acids and enzymesFurther, and can be expanded for multimodal bioreactors or theragnostic vehicles by additionally encapsulating varying hydrophilic substances into the cavity.

研究成果の概要（英文）：In this study, vesicular PICs were fabricated using a series of chemically modified SSOs or HDO and guanidinylated PEG-b- polypeptides for the stable codelivery of SSO with RNase H into cultured cells. The 100 nm sized vesicular structure was successfully stabilized in physiological milieu by a polyion pair of PS-Gapmer SSO and PEG-P(Asp-AG/G8O). Particularly, both single-stranded, PS backbone oligonucleotides and guanidinylated polypeptides are crucial for the stable nano- vesicle formation in the physiological milieu, presumably due to their enhanced noncovalent interactions, including hydro- phobicity and polyion-pairing capacity. The stabilized SSO- embedded nanovesicle (SSOsome) effectively encapsulated RNase H through physical entrapment using simple vortex- mixing. The RNase H-encapsulated SSOsome (H/SSOsome) enabled the efficient codelivery of SSO and RNase H into cultured cancer cells, leading to the significantly enhanced gene knockdown effect with negligible cytotoxicity.

研究分野：drug delivery

キーワード：polymeric vesicle　oligonucleotide　antisense　polyplex　encapsulation　RNase H　gene knockdown　codelivery

１．研究開始当初の背景

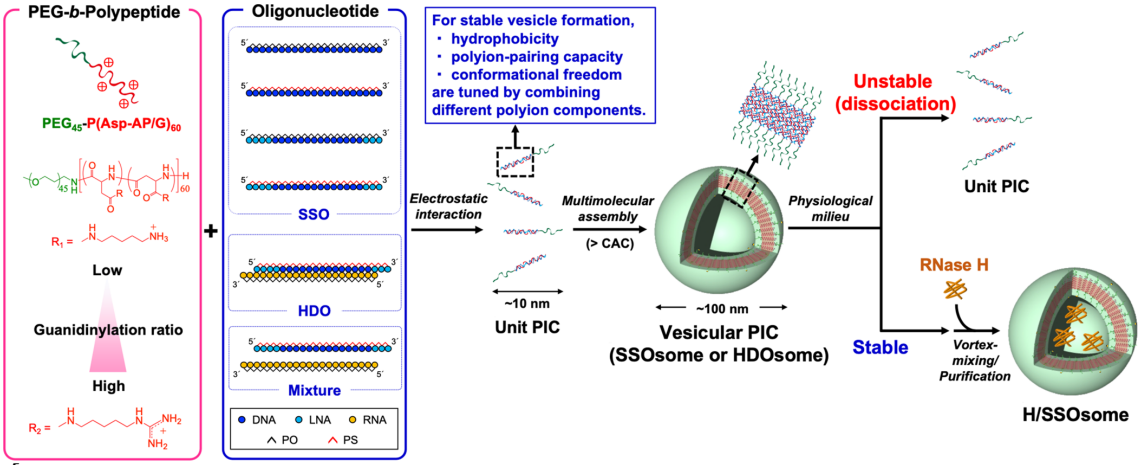
Recently, synergistic–cooperative biological events between small nucleic acids and their effector proteins, e.g., antisense effect (antisense oligonucleotide (ASO)+ribonuclease H (RNase H)), RNAi (siRNA+Ago2), and CRISPR-Cas (gRNA+Cas9), have been tremendously highlighted for the genome engineering as well as therapeutic applications. Particularly, the delivery technologies (or vehicles) have been extensively developed in the last decade. Nevertheless, there are few studies reporting on the simultaneous delivery of the two cooperative components because their varying physicochemical characteristics more likely hamper stable encapsulation into the single formulation.

２．研究の目的

Previously, we developed a nanovesicle (siRNAsome) using small nucleic acids, siRNA. The siRNAsome can co-loaded siRNA and another hydrophilic macromolecules in the membrane and interior, respectively. However, siRNAsomes have a dilemma for cross-linking that is necessary for stability but causes denaturation of encapsulated enzyme in the vesicles. Therefore, we focused on stable nanovesicles without cross-linking that affect enzymes.

３．研究の方法

The present study has tackled this issue by creating a novel nanovesicle possessing chemically modified single-stranded oligonucleotide (SSO; i.e., gapmer ASO with phosphorothioate (PS) linkage and locked nucleic acid (LNA)) in the vesicular membrane and RNase H in the vesicular cavity. Importantly, the careful design of nucleotide and polycation structures dramatically elevated the nanovesicle stability in the biological fluids. In this way, SSO and RNase H were successfully delivered into the target cells, allowing for the synergistically enhanced gene knockdown compared to the single delivery of SSO.

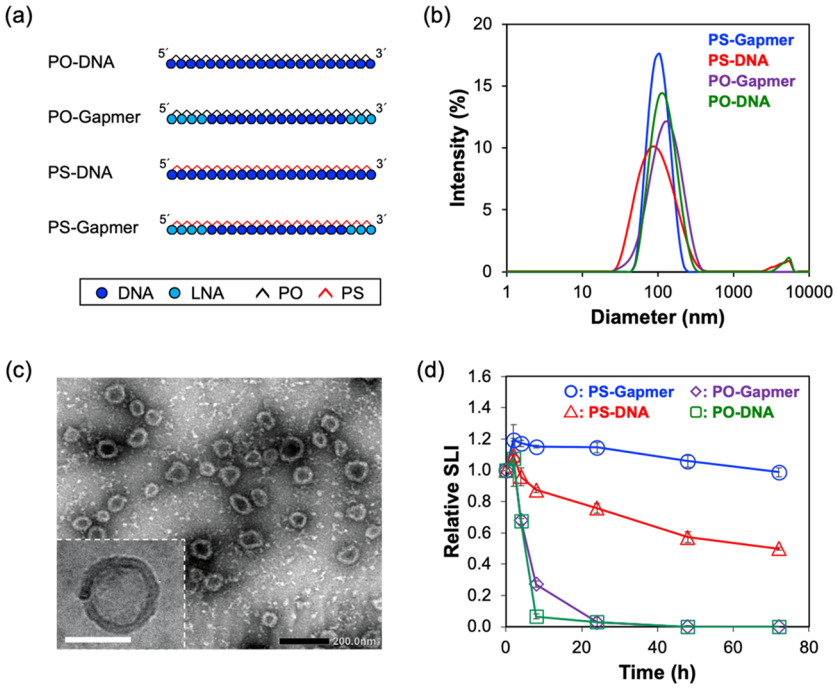


[Schematic Illustration Depicting the Fabrication of Noncovalently Stabilized Vesicular PICs Using Cationic PEG-b-polypeptides and Anionic Oligonucleotides]

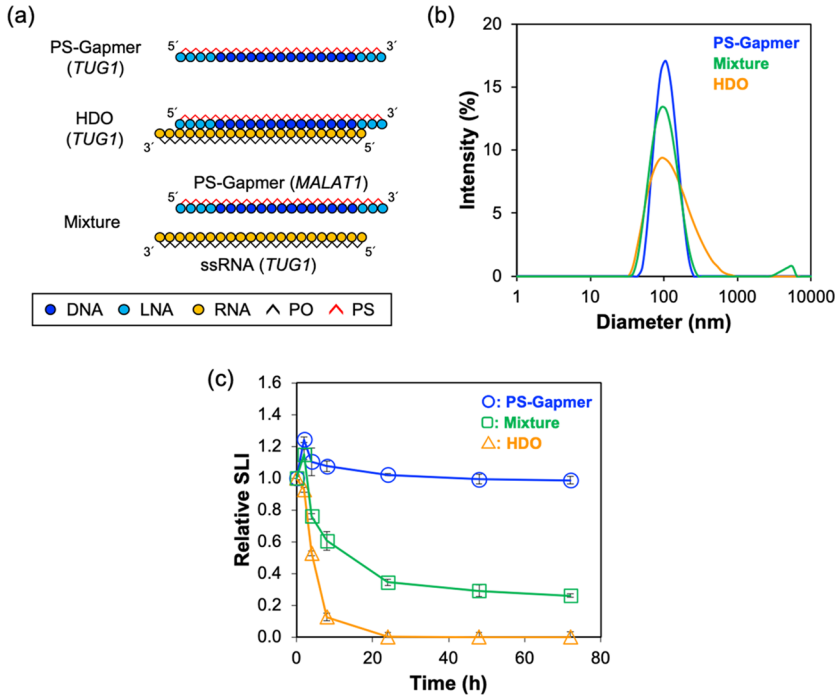
The polypeptide segment in PEG-b-polypeptide is guanidinylated to form salt bridges (or electrostatic and hydrogen-bonding interactions) with oligonucleotides. The oligonucleotides are designed to contain two varying chemical modifications, PS backbone and LNA termini, in the form of SSO or HDO to regulate the hydrophobicity, polyion-pairing capacity, and the conformational freedom of the PIC domain. The optimized polyion pair can accelerate the multimolecular association of unit PICs, directed toward the stable formation of vesicular PICs under physiological salt conditions. The stabilized vesicular PICs are further utilized for the intracavity encapsulation of RNase H to ensure the codelivery and cooperative gene knockdown in cells.

４．研究成果

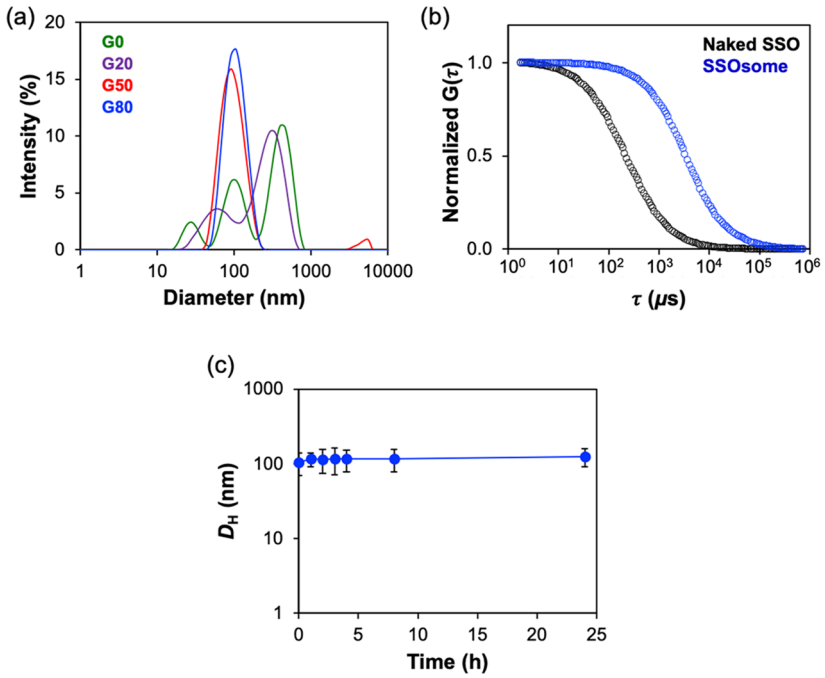
1. Fabrication of nanovesicles comprising the SSO-intercalated membrane at a substantially high loading capacity of SSO (~2,500 SSOs per vesicle)
2. Noncovalent stabilization of nanovesicles by focusing on the chemical structures of nucleotides in SSO as well as the polycationic segment in block copolymer
3. Codelivery of SSOs with their effector enzymes (RNase H) demonstrating the synergistic gene knockdown in cultured cells.



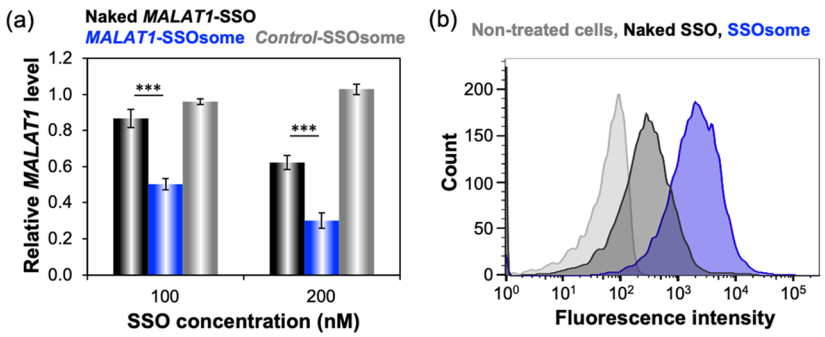
(a) Structural illustration of chemically modified SSOs. (b) Size distribution histograms of PICs prepared from PEG-P(Asp-AP/G80) and varying SSOs (base sequence: MALAT1) in 10 mM HEPES buffer (pH 7.3) without NaCl (SSO concentration: 10 μM). (c) Transmission electron microscopy (TEM) image of PICs prepared between PEG-P(Asp-AP/G80) and PS-Gapmer. Inset: cryogenic TEM image. The Black and white scale bars are 200 and 100 nm, respectively. (d) Time-dependent change in relative SLI of PICs prepared from PEG-P(Asp-AP/G80) and varying MALAT1-targeted SSOs in 10 mM HEPES buffer (pH 7.3) with 150 mM NaCl (SSO concentration: 5 μM).



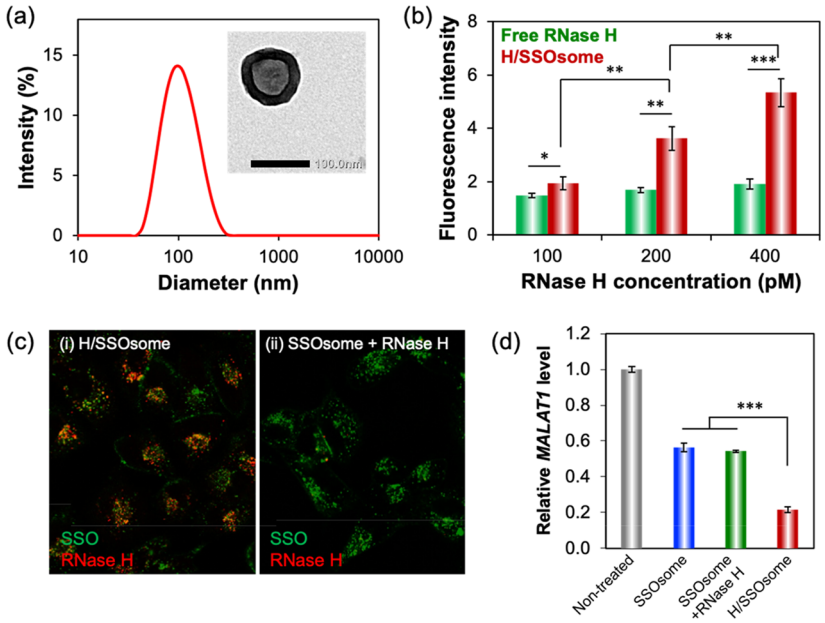
(a) Structural illustration of chemically modified SSOs and HDO. (b) Size distribution histograms of PICs prepared from PEG-P(Asp- AP/G80) and PS-Gapmer, Mixture, or HDO (base sequences: MALAT1 or TUG1) in 10 mM HEPES buffer (pH 7.3) without NaCl (oligonucleotide concentration: 10 μM). (c) Time-dependent change in relative SLI of PICs prepared using PEG-P(Asp-AP/G80) and PS- Gapmer, Mixture, or HDO in 10 mM HEPES buffer (pH 7.3) with 150 mM NaCl (oligonucleotide concentration: 5 μM).



Physicochemical characterization of PICs prepared from PS-Gapmer and PEG-P(Asp-AP/G)s. (a) Size distribution histograms of PICs prepared at 10 μM PS-Gapmer from PEG-P(Asp-Ap/G)s with varying degrees of guanidinylation in 10 mM HEPES buffer (pH 7.3). (b) Autocorrelation curves of naked AF647-SSO and AF647-SSOsome prepared from PEG-P(Asp-Ap/G80) at 1 μM AF647-SSO in 10 mM HEPES buffer (pH 7.3). (c) Time-dependent change in DH of SSOsome prepared from PEG-P(Asp-Ap/G80) at 0.5 μM AF647-SSO in a 150 mM NaCl/ 10% FBS-containing medium.



Biological activities of SSOsomes. (a) Gene knockdown efficiency determined by qRT-PCR. The cells were incubated with naked MALAT1-SSO or MALAT1/Control-SSOsomes for 48 h. (b) Cellular uptake efficiency determined by flow cytometry. The cells were incubated with free AF647-SSO or AF647-SSOsome at 200 nM SSO for 4 h.



Physicochemical and biological characterization of RNase H-encapsulated SSOsome (H/SSOsome). (a) Size distribution histogram of H/SSOsome at 7.5 μM SSO. Inset: TEM image of H/SSOsome. The scale bar is 100 nm. (b) Cellular uptake efficiency of RNase H determined using a fluorescent microplate reader. The cells were incubated with free AF647-RNase H or AF647-RNase H-encapsulated SSOsome (H/ SSOsome) for 6 h. (c) Confocal laser scanning microscopic images of A549 cells incubated with (i) H/SSOsome prepared from AF647-SSO and AF594-RNase H and (ii) a mixture of SSOsome and free AF594-RNase H (SSOsome + RNase H) for 24 h. AF647-SSO and AF594-RNase H are shown in green and red, respectively. (d) Gene knockdown efficiency determined using qRT-PCR. The cells were incubated with SSOsome, SSOsome + RNase H, or H/SSOsome at 100 nM MALAT1-PS-Gapmer SSO and 100 pM RNase H for 48 h.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関