Application note

Formulation of Peptide-Based Nanoparticles Using TAMARA

Robust microfluidic formulation of WRAP5 nanoparticles for siRNA and pDNA delivery.

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Abstract

Peptide-based nanoparticles (PBNs) are promising non-viral carriers for nucleic acid delivery, combining efficient cellular internalization with favorable biocompatibility. Among these systems, **WRAP5**, a W- and R-rich Amphipathic cell-penetrating Peptide, forms self-assembling nanoparticles with nucleic acids through electrostatic interactions, providing an alternative to conventional lipid-based delivery systems.

In this study, **WRAP5-based PBNs carrying siRNA or pDNA** were formulated using the **TAMARA** microfluidic system, which incorporates reusable microfluidic chips. Across 72 formulations, the effects of **flow rate ratio (FRR), total flow rate (TFR), mixer geometry (herringbone or baffle), and formulation volume** on nanoparticle size and polydispersity were investigated, alongside storage stability and *in vitro* biological activity.

TAMARA produced **small, highly homogeneous PBNs**, with mean sizes typically ranging from **50–70 nm and PDI < 0.22**, largely independent of the tested process parameters. pDNA-loaded PBNs remained **highly stable during storage at 4°C** for up to 70 days, while siRNA-loaded PBNs showed moderate size increases over time. Importantly, *in vitro* assays confirmed biological activity: **WRAP5:siRNA PBNs achieved ~50% CDK4 silencing in GIST-T1 cells**, while **WRAP5:pDNA PBNs mediated efficient mCHERRY expression in HeLa cells**, demonstrating robust microfluidic formulation of PBNs for functional nucleic acid delivery.

Introduction

Peptide-based nanoparticles (PBNs), particularly those formed from cell-penetrating peptides (CPPs), represent a promising non-viral nucleic acid delivery system due to their efficient cellular internalization and low immunogenicity. These carriers spontaneously assemble into nanoscale complexes, often driven by **electrostatic interactions between cationic residues within the peptide sequences and negatively charged nucleic acids**. Among these peptides, **WRAP** (W- and R-rich Amphipathic Peptide) was developed for efficient nucleic acid delivery [1], with **WRAP5** (LLRLLRWWWRLLRLL) identified as a leading CPP candidate. WRAP-based PBNs formulated with siRNA have demonstrated efficient cellular uptake [2] and effective knockdown of target proteins across diverse cell types [3]. Their delivery potential has also been validated in *in vivo* models [4], [5].

Successful nanoparticle development for nucleic acid delivery depends on controlling key formulation and process parameters that influence physicochemical properties, stability, and batch-to-batch reproducibility, ultimately affecting therapeutic efficacy. **Microfluidic formulation** offers a highly controlled approach to nanoparticle production by enabling precise adjustment of key parameters such as mixing speed and carrier-to-cargo ratio.

WRAP-based PBNs follow a fundamentally different assembly mechanism from conventional LNPs. Whereas LNP production typically relies on rapid mixing of an organic lipid phase with an aqueous phase to induce solvent exchange and self-assembly, WRAP5 peptides and nucleic acids are both readily soluble in aqueous media. Consequently, WRAP5-based PBNs can be formulated through **fully aqueous-to-aqueous mixing**, without the need for an organic phase or solvent-exchange step.

TAMARA (Inside Therapeutics) is a microfluidic nanoparticle formulation platform designed for **controlled and reproducible mixing**, supporting workflows from early-stage screening to *in vivo* studies. Its microfluidic chip integrates two distinct mixer architectures: a **staggered herringbone mixer**, which uses chevron-shaped grooves to promote chaotic advection for rapid laminar mixing, and a **baffle mixer**, which uses internal obstructions to generate vortices and recirculation at higher flow rates. The system enables different process parameters and mixing geometries to be explored within the same platform.

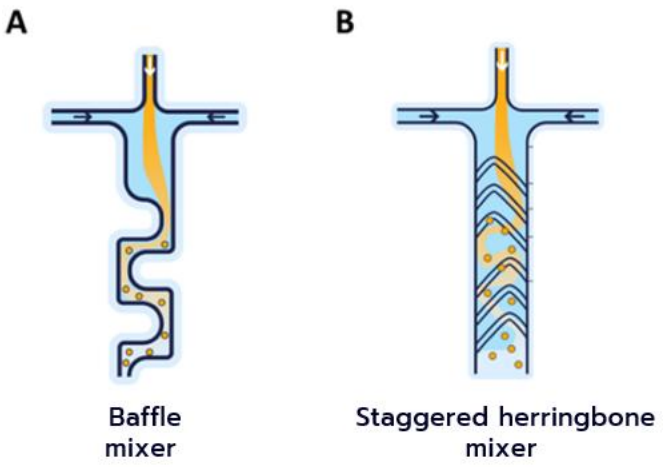

Figure 1. (A) *Baffle and* ***(B)*** *staggered herringbone mixer architectures integrated within the TAMARA microfluidic chip, enabling distinct microfluidic mixing mechanisms.*

In this study, the **TAMARA** Nanoparticle Formulation System was evaluated for the microfluidic production of **WRAP5-based PBNs carrying either small interfering RNA (siRNA) or plasmid DNA (pDNA)**. Two mixer geometries integrated within the TAMARA chip — **herringbone and baffle mixers** — were investigated alongside different **flow rate ratios (FRR) and total flow rates (TFR)** to determine their influence on PBN size and polydispersity. The robustness of the formulation approach was further investigated through **formulation upscaling and storage stability studies**. Finally, the biological activity of TAMARA-formulated PBNs was assessed *in vitro* for both nucleic acid cargos. **WRAP5:siRNA PBNs** were evaluated for cyclin-dependent kinase 4 (CDK4) silencing in GIST-T1 cells, while **WRAP5:pDNA PBNs** were evaluated for mCHERRY expression in HeLa cells. Together, this work investigates TAMARA as a robust and versatile microfluidic platform for peptide-based nucleic acid delivery, from systematic formulation and physicochemical characterization to *in vitro* biological performance.

This application note is based on work carried out at PhyMedExp, Inserm U1046, CNRS UMR 9214, University of Montpellier, and supported by Inside Therapeutics. A complete description of this study has been published as a rapid communication in the *Journal of Peptide Science* by Hammoum et al [6].

Results

1) Microfluidic production of WRAP5 PBNs using TAMARA: Effect of process parameters and formulation volume

WRAP5-based PBNs carrying siRNA or pDNA were formulated using TAMARA to investigate the influence of key microfluidic process parameters on nanoparticle size and homogeneity. Both herringbone and baffle mixers were evaluated across three FRRs (1:1, 3:1, and 5:1) and three TFRs (1.5, 5, and 8 mL/min). Both input streams consisted of 5% glucose solutions in water, one containing WRAP5 and the other containing the respective nucleic acid cargo. Each condition was tested in duplicate, resulting in 72 formulations, which were characterized by DLS for mean particle size and polydispersity index (PDI). Representative data for the selected FRR of 1:1 are summarized in Tables 1 and 2, including low-volume (0.5 mL) and upscaled (1.5 mL) formulations. Results from the complete process parameter screening across all FRRs and TFRs are reported in the original rapid communication [6] and its supplementary information.

For WRAP5:siRNA PBNs, formulated at a constant peptide:siRNA molar ratio of 20:1, mean sizes at day 0 ranged from approximately 55–67 nm, with PDI values of 0.18–0.22. Neither mixer geometry nor changes in FRR and TFR substantially affected particle size or homogeneity.

Table 1. Mean size and PDI of WRAP5:siRNA PBNs under selected formulation conditions on the day of formulation*. PBNs were produced using TAMARA with the baffle or herringbone mixer (FRR 1:1, TFR 5 mL/min) at low (0.5 mL) and increased (1.5 mL) formulation volumes, with manual formulation included for comparison at 1.5 mL. Adapted from Hammoum et al. [6]*

Formulation volume (mL)	Production method	Mean size (nm)	PDI
0.5	Baffle	57.2 ± 1.9	0.19 ± 0.02
	Herringbone	61.7 ± 1.2	0.18 ± 0.01
1.5	Baffle	63.4 ± 0.3	0.24 ± 0.01
	Herringbone	99.9 ± 0.7	0.17 ± 0.00
	Manual	77.5 ± 2.3	0.34 ± 0.02

Similarly, WRAP5:pDNA PBNs, formulated at a constant charge ratio of 3:1, showed mean sizes of approximately 48–57 nm and PDI values of 0.16–0.19 at day 0, with no observed dependence on the tested microfluidic conditions (FRR, TFR, mixer geometry).

Table 2. Mean size and PDI of WRAP5:pDNA PBNs under selected formulation conditions on the day of formulation*. PBNs were produced using TAMARA with the baffle or herringbone mixer (FRR 1:1, TFR 1.5 mL/min) at low (0.5 mL) and increased (1.5 mL) formulation volumes, with manual formulation included for comparison at 1.5 mL. Adapted from Hammoum et al. [6]*

Formulation volume (mL)	Production method	Mean size (nm)	PDI
0.5	Baffle	52.5 ± 0.5	0.16 ± 0.01
	Herringbone	47.9 ± 1.4	0.18 ± 0.03
1.5	Baffle	48.7 ± 1.3	0.20 ± 0.02
	Herringbone	49.9 ± 0.6	0.15 ± 0.00
	Manual	56.1 ± 1.9	0.22 ± 0.03

Based on the absence of an observed effect of FRR and TFR on PBN characteristics, an FRR of 1:1 was selected for upscaled (1.5 mL) formulations, together with a TFR of 5 mL/min for WRAP5:siRNA PBNs and 1.5 mL/min for WRAP5:pDNA PBNs. Increasing the formulation volume from 0.5 to 1.5 mL resulted in no important changes in particle size or PDI compared with the corresponding low-volume formulations. The upscaled PBNs also showed comparable colloidal characteristics to those prepared manually at 1.5 mL.

Together, these results demonstrate that **WRAP5 PBN formation is highly robust across the investigated microfluidic process parameter space, maintaining small particle sizes and low polydispersity for both siRNA and pDNA cargos despite changes in FRR, TFR, mixer architecture, and formulation volume**.

2) Storage stability of TAMARA-formulated WRAP5 PBNs

The storage stability of upscaled WRAP5:siRNA and WRAP5:pDNA PBNs produced using the herringbone and baffle mixers was evaluated at 4°C and compared with manually prepared formulations. Particle size and PDI were monitored by DLS at days 0, 7, and 55 for WRAP5:siRNA PBNs, and at days 0, 7, 48, and 70 for WRAP5:pDNA PBNs.

WRAP5:siRNA PBNs initially increased in mean size from approximately 60 to 100 nm after 7 days of storage, while maintaining PDI values below 0.25. Particle size subsequently remained around 100 nm up to day 55 for the herringbone and manually prepared formulations, indicating relatively stable colloidal properties after the initial size increase. In comparison,

PBNs produced using the baffle mixer exhibited lower long-term stability, with mean sizes exceeding 150 nm at extended storage times.

In contrast, WRAP5:pDNA PBNs exhibited high colloidal stability, with particle size and PDI remaining largely unchanged for up to 70 days at 4°C. Comparable profiles were observed among herringbone, baffle, and manually prepared formulations.

Overall, these findings show that **TAMARA-formulated WRAP5 PBNs retain their colloidal characteristics during storage at 4°C, with cargo-dependent differences in long-term stability and particularly high stability for pDNA-loaded PBNs**.

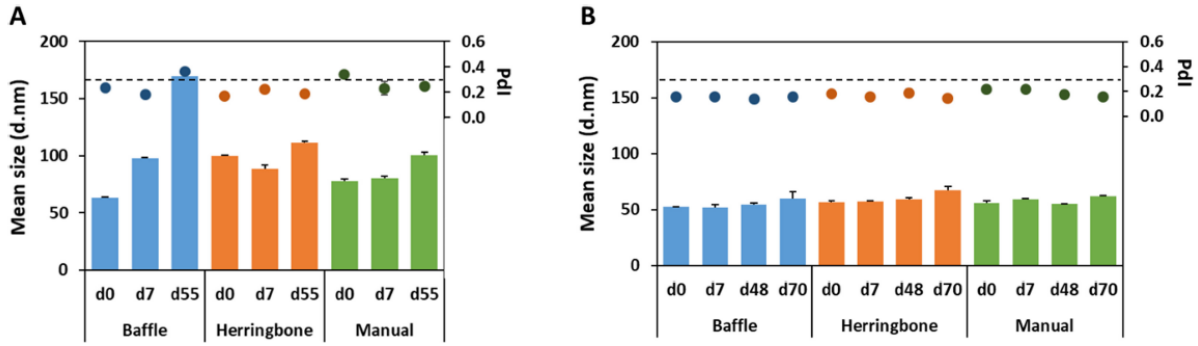

Figure 2. Storage stability of WRAP5 PBNs at 4°C. (A) *WRAP5:siRNA and* ***(B)*** *WRAP5:pDNA PBNs produced using TAMARA with the baffle or herringbone mixer, or by manual formulation. Particle size and PDI were monitored by DLS for up to 55 and 70 days, respectively. Reproduced from Hammoum et al. [6]*

3) Target protein knockdown by TAMARA-formulated WRAP5:siRNA PBNs

The biological activity of TAMARA-formulated WRAP5:siRNA PBNs was evaluated in human gastrointestinal stromal tumor (GIST-T1) cells using an siRNA targeting cyclin-dependent kinase 4 (CDK4). PBNs produced using the baffle or herringbone mixer at a TFR of 5 mL/min and FRRs of 1:1, 3:1, and 5:1 were assessed for CDK4 silencing by Western blot at a final siRNA concentration of 50 nM.

PBNs stored at 4°C for 14, 21, 29, or 55 days produced comparable levels of CDK4 knockdown across all tested FRRs and both mixer architectures. Overall, CDK4 expression was reduced by approximately 50% compared with non-treated cells, demonstrating that biological activity was retained during prolonged storage.

The upscaled WRAP5:siRNA PBNs, stored for 55 days, were subsequently compared across baffle, herringbone, and manual formulation methods, together with a freshly prepared manual formulation. All formulations maintained significant CDK4 silencing of approximately 50%,

confirming that activity was preserved following both microfluidic upscaling and long-term storage. Cell viability remained comparable to non-treated cells across conditions.

Overall, **TAMARA-formulated WRAP5:siRNA PBNs maintained consistent CDK4 silencing and cell viability across different process conditions and after prolonged storage at 4°C**.

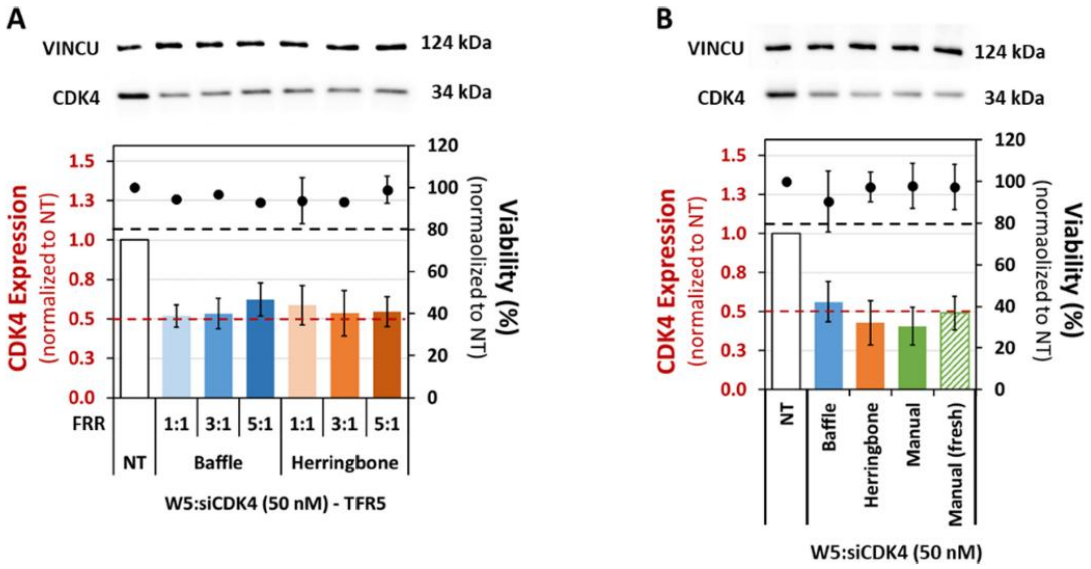

Figure 3. CDK4 silencing and cell viability following treatment with WRAP5:siRNA PBNs in GIST-T1 cells. ***(A)*** *PBNs produced using TAMARA with the baffle or herringbone mixer at different FRRs and stored at 4°C for up to 55 days (TFR 5 mL/min);* ***(B)*** *upscaled PBNs produced using either mixer and stored at 4°C for 55 days, compared with stored and freshly prepared manual formulations (TFR 5 mL/min, FRR 1:1). Reproduced from Hammoum et al. [6]*

4) Reporter gene expression by TAMARA-formulated WRAP5:pDNA PBNs

The biological activity of WRAP5:pDNA PBNs was evaluated in human cervical cancer (HeLa) cells using a plasmid encoding mCHERRY with a nuclear localization sequence (NLS). Upscaled PBNs produced using the baffle or herringbone mixer (FRR 1:1, TFR 1.5 mL/min) were stored at 4°C for 48 days and compared with manually prepared formulations. Cells were transfected with a final pDNA quantity of 1 µg, and mCHERRY expression was assessed by confocal fluorescence microscopy after 24 h.

Quantification of the nuclear mCHERRY signal showed expression in more than 35% of HeLa cells, with comparable levels observed for PBNs produced using the baffle, herringbone, or manual formulation methods. In addition to nuclear localization, mCHERRY fluorescence was observed in the cytoplasm, likely corresponding to newly translated protein that had not yet

translocated to the nucleus. As quantification was based on the nuclear signal, the measured percentage may therefore underestimate the overall transfection efficiency.

Overall, **WRAP5:pDNA PBNs retained their transfection activity after 48 days of storage, with comparable mCHERRY expression across microfluidic and manual formulation methods**.

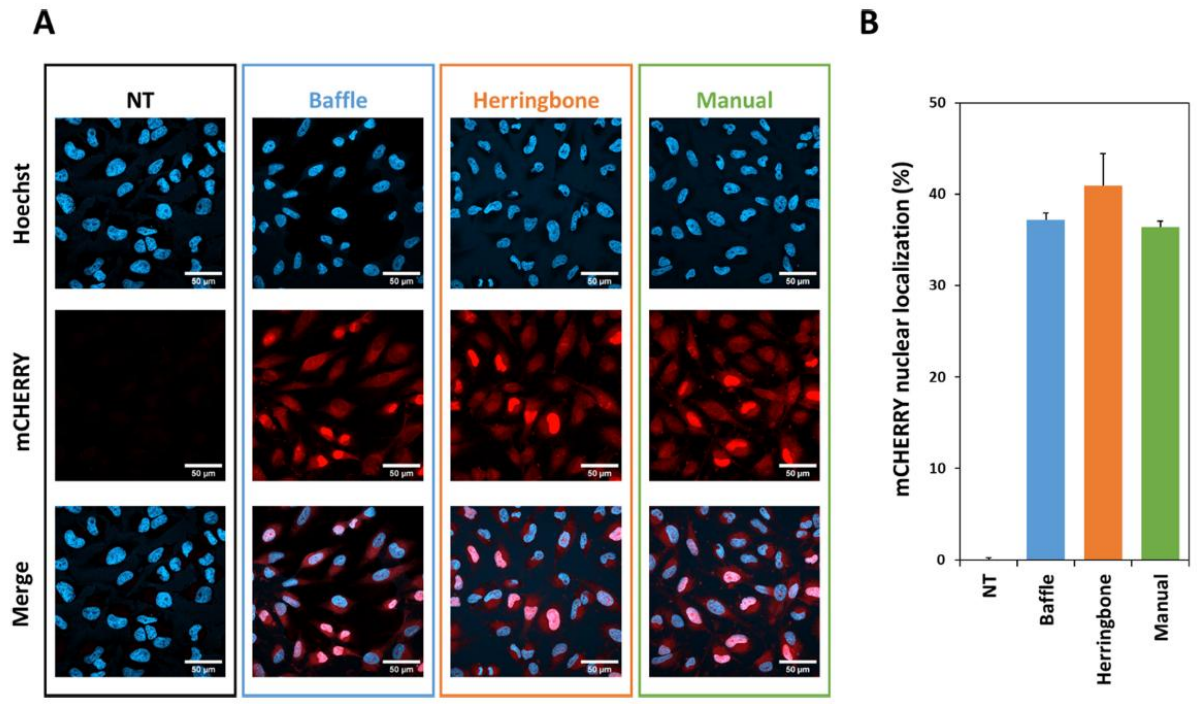

Figure 4. mCHERRY expression mediated by WRAP5:pDNA PBNs in HeLa cells. (A) *Representative confocal fluorescence images following transfection with upscaled PBNs produced using the baffle or herringbone mixer (TFR 1.5 mL/min, FRR 1:1) and stored for 48 days at 4°C, compared with manual formulations and non-treated cells (NT). Blue: Hoechst; red: mCHERRY. Scale bar: 50 µm.* ***(B)*** *Quantification of nuclear mCHERRY expression normalized to the Hoechst nuclear signal. Reproduced from Hammoum et al. [6]*

Conclusion

This study demonstrates the **microfluidic production of WRAP5-based PBNs through fully aqueous-to-aqueous mixing**, without the solvent-exchange step typically used for lipid nanoparticles. Using TAMARA, PBNs carrying either **siRNA or pDNA** were reproducibly produced with small particle sizes and low PDI values.

Importantly, **PBN characteristics were largely insensitive to the investigated FRR, TFR, and mixer architecture**, demonstrating a broad microfluidic operating window with limited need for process optimization. **Increasing the formulation volume** from 0.5 to 1.5 mL also maintained

PBN physicochemical properties, with TAMARA-produced formulations showing characteristics comparable to manual preparation.

The resulting **PBNs remained stable during storage**, particularly pDNA-loaded PBNs, which maintained their colloidal properties for up to 70 days at 4°C. Most importantly, **both WRAP5:siRNA and WRAP5:pDNA PBNs retained their biological activity** following prolonged storage.

Overall, TAMARA provides a robust and reproducible platform for aqueous microfluidic production of functional WRAP5 PBNs for both siRNA and pDNA delivery.

Material and methods

1) Formulation of WRAP5 PBNs using TAMARA

WRAP5-based PBNs carrying either siRNA or pDNA were produced using TAMARA (Inside Therapeutics). All formulations were prepared in a filtered aqueous solution of 5% (w/v) glucose, allowing fully aqueous-to-aqueous microfluidic mixing without an organic solvent or solvent-exchange step. The WRAP5 peptide and nucleic acid cargo were prepared separately in the same glucose solution and introduced through the two TAMARA inlet streams.

Herringbone and baffle mixer architectures of the cyclic olefin copolymer (COC) microfluidic chips were evaluated. Formulations were produced at room temperature under a constant input pressure of 7 bar. As a 5% glucose solution has a higher viscosity than water, its physicochemical properties were incorporated into the TAMARA settings (dynamic viscosity ~1,200 µPa·s at 20°C; temperature sensitivity ~30 µPa·s/°C; solvent molar volume 18.5 cm^3/mol), and temperature was controlled throughout formulation.

The influence of microfluidic conditions was investigated using FRRs of 1:1, 3:1, and 5:1 and TFRs of 1.5, 5, and 8 mL/min with both mixer architectures. Formulations were initially produced at 500 µL, with selected conditions subsequently increased to 1,500 µL. For WRAP5:siRNA PBNs, a constant peptide:siRNA molar ratio (MR) of 20:1 was maintained across all conditions. For WRAP5:pDNA PBNs, a constant charge ratio (CR) of 3:1 was maintained.

For subsequent increased-volume experiments, an FRR of 1:1 was used with a TFR of 5 mL/min for WRAP5:siRNA and 1.5 mL/min for WRAP5:pDNA formulations. Corresponding manual formulations were prepared by volume-to-volume mixing at an FRR of 1:1 while maintaining the same MR or CR. Between runs, reusable chips were cleaned using the integrated TAMARA cleaning procedure (up to 8-times).

2) Characterization and storage stability of WRAP5 PBNs

Mean hydrodynamic diameter and PDI were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments) at 25°C and a backscattering angle of 173°. Measurements were performed in triplicate. For stability studies, PBNs were stored at 4°C and characterized over time: WRAP5:siRNA PBNs at days 0, 7, and 55, and WRAP5:pDNA PBNs at days 0, 7, 48, and 70.

3) Cell culture and biological activity

Human gastrointestinal stromal tumor (GIST-T1) cells and human cervical cancer (HeLa) cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO_2.

For siRNA activity, GIST-T1 cells were treated with WRAP5:siCDK4 PBNs at a final siRNA concentration of 50 nM for 1.5 h under serum-free conditions, followed by 24 h incubation in serum-containing medium. CDK4 protein levels were assessed by Western blotting, using vinculin as a loading control, and quantified using Fiji/ImageJ. Cell viability was evaluated using an LDH cytotoxicity assay.

For pDNA activity, HeLa cells were treated with WRAP5:pDNA PBNs containing 1 µg mCHERRY pDNA for 1.5 h under serum-free conditions, followed by 24 h in the presence of serum. Cells were fixed, nuclei were stained with Hoechst, and mCHERRY expression was visualized using a Zeiss LSM800 confocal microscope. Nuclear mCHERRY fluorescence was quantified relative to the Hoechst signal using Fiji/ImageJ.

For further details on the experimental protocols and methods, please refer to the supplementary information of the published rapid communication [6].

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