

Conjugation of Hydrophobic Drugs to Motile pRNA 4WJ Nanoparticles for Spontaneous Tumor Targeting and Undetectable Toxicity

Daniel W Binzel,^{1,*} Kai Jin,¹ Yudhistira Tesla,¹ and Peixuan Guo^{1,2,*}

¹Center for RNA Nanobiotechnology and Nanomedicine; Division of Pharmaceutics and Pharmacology, College of Pharmacy; and Comprehensive Cancer Center. The Ohio State University, Columbus, Ohio 43210, USA

²Comprehensive Cancer Center, College of Medicine. The Ohio State University, Columbus, Ohio 43210, USA

Editorial summary: This protocol describes the synthesis, purification, and assembly of stable branched 4-way junction (4WJ) RNA nanoparticles conjugated with multiple hydrophobic chemotherapeutic drug molecules to target specific tumors with minimal side effects.

Tweet: Synthesis and assembly of 4WJ RNA nanoparticles for targeted drug delivery to tumors.

Cover teaser: Synthesis of 4WJ RNA nanoparticles for tumor targeted drug delivery

Key points:

- RNA strands containing modified nucleotides are produced using solid phase synthesis for conjugation with multiple chemotherapeutic prodrugs, including Camptothecin, Paclitaxel and SN38, by copper chelated Click chemistry prior to assembling the 4WJ RNA nanoparticles, which can be loaded with up to 24 drug molecules per nanoparticle. Copper was removed from the RNA 4WJ complex during purification steps due to the hundreds-fold size difference.
- The RNA nanoparticles can be designed to contain a tumor-targeting ligand, resulting in specific uptake by the target cell type, while the drug payload is released via cleavage by esterases in the tumor microenvironment to ensure site-specific delivery.

Key references:

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3. Li, X. *et al.* RNA four-way junction (4WJ) for spontaneous cancer-targeting, effective tumor-regression, metastasis suppression, fast renal excretion and undetectable toxicity. *Biomaterials* 305, 122432, doi:10.1016/j.biomaterials.2023.122432 (2023).

***Correspondence to:**

Peixuan Guo

Email: guo.1091@osu.edu

or

Daniel W. Binzel

Email: binzel.2@osu.edu

[H1] ABSTRACT

Chemotherapeutics are widely used in cancer treatments, but their toxicity, bioavailability, and solubility have been critical topics. RNA nanotechnology has emerged as a promising modality for targeted delivery of chemotherapeutics. Structurally, RNA is thermostable with high melting temperature (T_m), while conformationally it is dynamic and flexible. RNA's unique deformability and motility lead to rapid spontaneous tumor accumulation and glomerular excretion, thus fast body clearance; while its anionic charge and favorable size prevents accumulation in vital organs for undetectable toxicity. We developed branched 4WJ (4-way junction) nanoparticles that were stable with a T_m above 80 °C, even when conjugated with drugs. Each 4WJ RNA component strand can conjugate 6 molecules of drug, such as Camptothecin, Paclitaxel, and SN-38. Thus, each 4WJ carries a total of 24 molecules of hydrophobic chemical drugs spaced to prevent aggregation. RNA conjugation improved drug water solubility by 32,000-fold. This protocol describes the construction of 4WJ RNA drug complexes for cancer therapy. Specific procedures include the modification of chemical drugs, conjugation of multiple prodrug molecules to each RNA component strand, assembly of RNA nanoparticles, and their purification and characterization. Chemical drugs are conjugated to RNA nanoparticles via efficient click chemistry, creating an ester linker that is in tumor tissues or tumor cells. Upon delivery and release, the prodrugs returns back to their original structures and chemistry. Inclusion of tumor targeting ligands demonstrated specific delivery of high payload chemotherapeutics to tumors, controlled release of chemical drugs, and strong tumor inhibition.

INTRODUCTION

Chemotherapeutics are a popular and effective method in cancer therapy, since small molecules can enter and inhibit rapidly dividing cells with fast metabolic processes. However, many chemotherapeutics face the challenges of unfavorable bioavailability, poor cellular transport, and indiscriminate targeting of cells. As a result, patients treated with chemotherapeutics suffer from high levels of toxicity to healthy organs and poorly tolerate delivered doses. Chemotherapeutics have undergone reformulations into less-toxic prodrugs or nanoparticle conjugates by enhancing specific delivery and reducing toxicity.¹⁻⁴ However, the development of chemoresistance and recurrence in late stage in cancer has become a significant problem.^{3,5,6} There is a great need for on-tumor delivery of chemotherapeutics that can overcome the above-mentioned limitations. Antibody-drug conjugates (ADC) have recently come to the market and specifically bind therapeutics targets on tumors, while also delivering chemotherapeutic cargos or conjugates. This on-target delivery has generated great interest with over 240 clinical trials in 2024.⁷⁻⁹ ADCs have greatly advanced drug delivery but due to the amphiphilic nature of proteins and their relatively larger size, they can generate side effects and toxicities from immune responses, they suffer from early release of chemotherapeutics and are prone to aggregation.¹⁰

The discovery of a small noncoding packaging RNA (pRNA) in the genome of the bacteriophage phi29,¹¹ and the special hand-in-hand interactions¹² that form hexameric rings led to the emergence of RNA nanotechnology.¹³ It was previously predicted that RNA therapeutics would be the third milestone in pharmaceutical drug development.¹⁴ The clinical application of RNA has rapidly gained interest following successful development of mRNA vaccines during the SARS-CoV-2 pandemic¹⁵⁻¹⁷ along with the FDA approval of *Onpattro* as the first RNA therapeutic followed by approval of subsequent therapeutics.¹⁸ Within RNA therapeutics, RNA nanotechnology has developed into a promising field that has demonstrated both spontaneous and active targeting to cancer tumors in mouse models.¹⁹⁻²¹ RNA nanoparticles self-assemble from several short RNA oligos into stable and branched motifs;¹³ allowing for functionalization through conjugation of additional groups such as RNA aptamer or chemical targeting ligands,²²⁻²⁷ interfering RNA (RNAi) (including siRNA, miRNA, and anti-miRNA),²⁸⁻³⁴ fluorescent or radioactive markers,^{27,35-37} ribozymes,^{22,38} and chemotherapeutics.³⁹⁻⁴² RNA nanoparticles, including their core motif and functional groups, are primarily composed of RNA and their design has been discussed at length in several high-profile reviews.^{13,19,43-45} RNA nanoparticles are motile and deformative allowing for their rapid and spontaneous accumulation in tumors, as the RNA nanoparticle can deform and squeeze through leaky tumor vasculature.¹⁹⁻²¹ Additionally, these properties allow for rapid renal clearance of non-tumor accumulated nanoparticles, resulting in safe biodistribution profiles.^{20,46} The addition of RNA aptamers that target tumor associated receptors allows for active tumor targeting of tumors and endocytosis into tumor cells for efficient cargo delivery.^{29-33,47} Finally, the immunogenicity of RNA nanoparticles is tunable by modulating nanoparticle sequence, size, shape, and stoichiometry allowing for the generation of safe RNA nanoparticles with no immune response.⁴⁸⁻⁵¹

[H2] Development of the protocol

The feasibility of RNA nanotechnology was first proven through the controlled assembly of nanorings composed of pRNA from the Phi29 DNA packaging motor.¹² This formation of RNA rings composed of multiple RNA subunits led to the idea of developing RNA nanoparticles for therapeutic applications.⁵² The Phi29 pRNA hexameric ring was conceptualized to carry a

functional module off each pRNA and proven as a functional nanoparticle through the combination of one pRNA harboring a cell targeting ligand and a second pRNA extended to include a siRNA.^{28,52,53} Through the years new RNA nanoscaffolds were developed to greatly improve the branched structuring for including more functional modules and drastically improve the enzymatic and thermodynamic stabilities.^{38,44,48,54-66} Thus, RNA nanoparticles have demonstrated the ability to serve as a therapeutic platform delivering nucleic acid-based therapies including siRNA, miRNA, and anti-miRNA. We have previously published a protocol on the development of RNA nanoparticles for the delivery of therapeutics RNAs.^{67,68} The resulting RNA nanoparticles were able to accumulate in tumor microenvironments and successfully delivered functional modules to silence specific genes resulting in tumor growth inhibition.^{29,30,32,33,54,69-74}

Recently, RNA nanoparticles were designed for the purpose of conjugating and solubilizing hydrophobic chemical drugs, including deliver paclitaxel (PTX), camptothecin (CPT), and SN38.³⁹⁻⁴² Since unusual thermostability of the phi29 pRNA three-way junction (3WJ) with high melting temperature (T_m) was reported,⁵⁴ branched RNA nanoparticles have been extensively investigated for their potential for RNAi and chemical drug delivery.³⁹ We developed a RNA four-way junction (4WJ), derived from the phi29 pRNA-3WJ. The resulting 4WJ holds a higher thermodynamic stability that can carry more chemical drugs. This 4WJ was found to form quickly at a high annealing temperature (T_a) of 80.9°C compared to 58.4°C of the smaller pRNA-3WJ and resulted in increased drug payload on 24 molecules compared to only 10 molecules of the pRNA-3WJ.³⁹ The 4WJ nanoparticle is a rather small nanoparticle, slightly smaller than 10 nm and simplistic in that it is composed of 4 short (~40 nt) oligo strands which rely on base pairing to form its highly stable structure. Due to the unique capabilities of the 4WJ nanoparticle, paclitaxel conjugation resulted in enhancing the drug solubility by 32,000-fold.³⁹ The resulting 4WJ RNA drug complex spontaneously accumulated in tumors within 30 min after IV administration, even without ligands.^{20,37} For non-tumor accumulated nanoparticles, fast renal excretion and rapid body clearance results in little organ accumulation, undetectable toxicity or immunogenicity. The safety parameters such as organ histology, blood biochemistry, and pathological analysis have been reported.⁴² The highly efficient cancer inhibition, undetectable toxicity, and favorable CMC production of these RNA nanoparticles generates a candidate with high potential for translation in cancer therapy. As such, RNA nanoparticles have a bright future in the pharmaceutical field and are part of the now third milestone in pharmaceutical drug development.¹⁴

The transition of producing RNA nanoparticles via solid-phase chemical synthesis from *in vitro* transcription allowed for the inclusion of chemically modified nucleotides within RNA nanoparticles. The available wide array of chemically modified phosphoramidites allow for production of 4WJ with diverse nucleotide structures^{39-41,61} as compared to those that were produced by biological RNA polymerases.⁷⁵⁻⁷⁸ Click chemistry, first developed by K.B. Sharpless,^{79,80} provides simple and efficient chemical reactions that are easily adaptable for linking biological molecules through the use of organic chemical groups. The approaches via solid-phase synthesis using modified phosphoramidites, such as including the alkyne modifications, make it possible to apply the highly efficient Click chemistry to RNA nanotechnology. For example, the 2'-propargyl modifications can be achieved for multiple nucleotides in the RNA strand.^{39,41} Click chemistry simplified synthetic chemistry techniques needed for the conjugation of chemotherapeutic prodrugs to RNA oligos while maintaining a high yield and reaction selectivity. The azide alkyne Huisgen cycloaddition reaction,^{79,81} used in this protocol, provided a feasible drug conjugation to RNA oligos in a single step.

This protocol presents the development of thermodynamically and enzymatically stable 4WJ RNA nanoparticles capable of targeting tumors and delivering conjugated chemotherapeutics safely and effectively.^{39,41} Developed 4WJs are synthesized with 24 molecules of various chemotherapeutic prodrugs, including PTX, CPT, and SN38.³⁹⁻⁴¹ The high tumor accumulation of the nanoparticles results in specific chemotherapeutic release of prodrugs into authentic drugs as their original chemical structure in the tumor microenvironment by tumor associated esterases and diminishes the toxicity and side effects of the delivered chemotherapeutics.⁴²

[H2] Overview of the Procedure

This protocol covers steps for producing thermostable 4WJ RNA nanoparticles with hydrophobic chemical drugs and characterizing the nanoparticles' biophysical characteristics and anti-cancer efficacy. The protocol covers the synthesis of RNA oligos, production of chemotherapeutic prodrugs, conjugation of prodrugs to RNA, and finally the assembly and purification of RNA 4WJ nanoparticles (**Fig. 1**). A flow chart of the procedure highlighting the estimated timing of each stage is provided in **Fig. 2**.

The production of RNA nanoparticles conjugated with chemical drugs begins with the design of the RNA nanoparticle itself. RNA nanoparticles of various sizes, shapes, and sequences have been constructed to include branched motifs (3WJ,^{54,82-84} 4WJ,^{39,41,85,86} six-way junction [6WJ],³⁴ etc.), 2D planar geometric shapes (triangle, square, and pentagon),^{48,58,87-90} 3D shapes (tetrahedron, prisms, and dodecahedron),^{57,91-93} and micellular structures.^{61,94} These various nanoparticles are highlighted in our previous *Nature Protocols* and *Chemical Reviews* publications.^{19,67} For conjugation of chemotherapeutics, it is important to ensure the RNA nanoparticle remains stable following drug conjugation while preventing drug aggregation.³⁹ The conjugation of chemotherapeutics can affect the base pairing of the nucleotides resulting in low thermostability of the nanoparticle. As an example, the conjugation of paclitaxel to the phi29 pRNA-3WJ resulted in greatly reducing the thermostability of the normally stable 3WJ.³⁹ For this requirement, RNA nanoparticle design must be taken into consideration to ensure high thermostability. Furthermore, high G:C content of RNA has been commonly believed to be more thermodynamically stable, and one would consider mutating an RNA junction's sequence to improve thermostability. However, such mutations were found to be detrimental to the junction's structuring as demonstrated by Li *et al.*⁴¹ Additionally, the RNA nanoparticle must be designed to have space between drug conjugation sites by at least 5 nucleotides to ensure drugs do not aggregate and do not alter the 4WJ folding, while the strand-sequence must be long enough to allow for high drug loading.^{39,41} The RNA 4WJ used in this protocol is derived from phi29 pRNA-3WJ (**Fig. 3A**).^{39,54} The 4WJ carries 24 drug conjugation sites while maintaining proper drug spacing. The high thermostability of the 4WJ is critical for maintaining stability upon drug conjugation. An additional nanoparticle design component is the inclusion of fluorophore(s) for tracking RNA targeting and distribution;³⁵ or cancer targeting ligands, such as Folate,^{52,54} EGFR RNA aptamer,⁹⁵ and EpCAM RNA aptamer.⁹⁶

Next, RNA oligos that compose the 4WJ RNA nanoparticle must be produced. Synthesis of RNA can generally occur via *in vitro* transcription using a T7 polymerase, or via solid-phase chemical synthesis. Each method has its advantages; however, chemical synthesis is required in this protocol as it allows for the use of chemically modified nucleotide phosphoramidites to incorporate chemical moieties used for drug conjugation (**Fig. 1**, step 1).³⁹⁻⁴² Each of the four oligo strands is synthesized to include six 2'-propargyl modified nucleotides to provide alkyne groups

used later in click chemistry conjugation of chemotherapeutic prodrug (**Fig. 1**, step 23). Synthesized RNA strands are purified by standard desalting procedures, reverse-phase high performance liquid chromatography (HPLC), or polyacrylamide gel purification. Desalting (described in the Procedure) provides the fastest procedure but at the cost of your RNA product containing some aborted strand contaminants; on the other hand, HPLC and gel purification are more time consuming but will provide RNA products of the exact desired size and thus sequence. Due to the optimal length and high affinity of the 4WJ component strands towards each other, generally the aborted strands are incorporated in a relatively low rate due to lowered affinity with other 4WJ strands.

Additionally, the desired chemotherapeutic must be selected. In this protocol, the conjugation of paclitaxel,^{39,61} camptothecin,⁴⁰ and SN38^{41,42} are demonstrated (**Fig. 4A**). All of these chemotherapeutics are highly effective in inhibiting cell growth, including many cancer cell types, and have been used in the clinic but have lost favor due to their limitations discussed above.⁹⁷⁻¹⁰¹ These drugs were also selected as they have a hydroxyl group that can be modified to generate a prodrug with an azide. The chemotherapeutic prodrugs were generated by Steglich esterification combining each of the chemotherapeutics with a 6-azido hexanoic acid to generate an azide linker (**Fig. 4B** for reaction scheme).^{40,102} The selection of an ester linker provides a stimuli-sensitive linker that is aimed to release the chemical drug in the presence of tumor esterases and return the prodrug back to its original structure. This prodrug is then purified by silica-column and characterized by, mass spectroscopy, thin-layer chromatography, and NMR to ensure the Steglich esterification reaction takes place and quantify the relative yield of the reaction. The synthesized RNA oligos are then reacted with these chemotherapeutic prodrug under copper chelated Click chemistry to link the azide to the RNA-alkyne (**Fig. 1** step 23).^{39,40} Copper chelated Click chemistry allows for simple and fast reaction for a high yield of drug conjugation to RNA. Additionally, Click chemistry utilizes biological molecules in a single reaction step (reaction scheme shown in **Fig. 5**). Following conjugation, the RNA strands are purified to remove excess unconjugated drug and copper via reverse-phase HPLC or polyacrylamide gel and characterized for drug incorporation.

The final stage of the procedure is the assembly of the RNA nanoparticle from each of its component strands (**Fig. 1**, step 32) and testing the delivery of the chemotherapeutic by the RNA nanoparticle. The 4WJ is assembled from the 4 strands followed by characterization to ensure assembly, size, and stability of the nanoparticle.³⁹ The RNA nanoparticles should be characterized for thermostability, enzymatic stability, drug release profiles, and nanoparticle size and charge characterization. The RNA nanoparticle drug conjugate is then tested in *in vitro* and *in vivo* cancer models to ensure safety and tumor inhibition. It is expected that the chemotherapeutic is released via the ester bond by either tumor esterases or water hydrolysis and returned to its original structure with the hydroxyl group.

[H2] Alternative methods

The concept of delivering toxic chemical drugs such as chemotherapeutics has been a long-term goal of nanotechnology.¹⁰³ Numerous nanoparticle material platforms that have loaded chemotherapeutics for the delivery to solid tumors have been developed. Below we discuss several alternative nanoparticle platforms.

[H3] Liposomes: Liposomes, a class of lipid based nanoparticles, since their discovery in 1965 have been used to deliver various therapeutic cargos through encapsulation into either the lipid bilayer or aqueous core.^{104,105} Liposomes are primarily made up of phospholipids and

include cholesterol which is essential for the particle formation and stability.^{105,106} Liposomes commonly also include PEGylated lipids to aid in stability and improve biodistribution profiles to aid in drug delivery. Loading of drugs is achieved by encapsulation during the formation of liposomes and various therapeutics can be loaded into their core, including hydrophilic drugs, RNAs, DNA, or proteins;¹⁰⁶ while hydrophobic drugs can be loaded into the lipid bilayer.¹⁰⁷⁻¹⁰⁹ Theoretically, liposomes can range from 20 to 1000 nm in size,¹⁰⁵ but are typically larger in size (<100 nm) which can cause macrophage phagocytosis, endoplasmic reticulum stress, and biodistribution challenges, due to lipids' ability to pass the cell membrane of healthy cells, as demonstrated by high levels of accumulation in healthy organs.¹¹⁰⁻¹¹² Liposomes on their own rely on passive tumor targeting, unless functionalized with ligands through additional assembly steps, thus limiting tumor accumulation and delivery to targeted cells.¹¹³ However, they benefit from being bio-degradable making them relatively safe and are able to release therapeutics without endosome trapping.¹⁰⁷ As a result, chemotherapeutics encapsulated in liposomes result in extended circulation time and extended release of drugs and are currently used in the clinic.¹¹⁴

[H3] Polymer based nanoparticles: Both simple and highly complex polymeric nanoparticles involving several polymers and layers have been designed.¹¹⁵⁻¹¹⁸ Polymeric nanoparticles can be composed of various polymers, but commonly use poly(lactic-co-glycolic acid) (PLGA) and are functionalized with polyethylene glycol (PEG). They are highly tunable for defined stability based on the polymers used and can vary in characteristics such as size, surface charge, and drug loading. Polymer nanoparticles inherently do not have cell specific targeting but have been modified to include tumor targeting ligands.^{115,116} Overall, polymer nanoparticles can suffer from homogeneity issues, accumulation in healthy organs due to their larger size, and lack of biocompatibility.^{115,119} Yet polymer nanoparticles have shown promise in shielding the loaded drug to prevent cytotoxicities and better solubilize drugs.

[H3] Inorganic nanoparticles: Inorganic nanoparticle make up a wide range of nanoparticles including iron oxide nanoparticles, ceramic nanoparticles, titanium, gold, silica, and silver nanoparticles.¹¹⁹⁻¹²¹ These nanoparticles typically comprise a solid core with functionalization on the exterior. These nanoparticles are commonly signal responsive, for example to photothermal therapy, or are photoresponsive at the delivery environment.¹²¹ This provides a precise release of therapeutics at the desired site, but inorganic nanoparticles often come with high accumulation in the lung, liver, and kidneys; require surface modifications for improved biodistribution;¹²² and result in toxicities.

[H3] Albumin based nanoparticles: Albumin is a natural ligand-carrying protein in the blood that has been utilized to load chemotherapeutics.¹²³⁻¹²⁵ The most notable albumin/drug complex is Abraxane or nab-paclitaxel,^{126,127} a paclitaxel loaded nanoparticle to enhance the drug solubility. Albumin nanoparticles vastly prolong the half-life of rapidly cleared drugs by enhancing the size of the chemical drugs to improve biodistribution. It is able to load high loads of drugs into a single protein.¹²³ However, the lack of effective tumor targeting has limited albumin nanoparticles and there is now criticism that Abraxane is unable to improve the toxicity and side-effects of paclitaxel, bringing question of its effectiveness.^{128,129}

[H3] Antibody-drug conjugates: ADCs have boomed onto the market, with nearly 100 entering clinical trials,¹³⁰ for their ability to provide on-target delivery of conjugated chemotherapeutics.^{7,8,131} ADCs are engineered monoclonal antibodies that have been designed to bind to an overexpressed receptor on tumors to allow for active tumor targeting while carrying chemotherapeutics that are conjugated through linkers similar to those used on RNA

nanoparticles. Additionally, ADCs can provide a secondary therapeutic response through receptor inhibition on the tumor cells.¹³⁰ The construction of ADCs requires several steps that make their production complex compared to other nanotechnology platforms. The amphophilic nature and bulky size of ADCs can result in nonspecific cell binding and unfavorable biodistribution. However, their efficacy outweighs these negatives. Furthermore, a current challenge in the ADC field is overcoming toxicities, such as central nervous system toxicity, hypersensitivity reactions, and immune responses.^{10,130} These may be related to ADCs being proteins and thus eliciting immune responses, and issues with homogeneity and stability of drug conjugates.

[H3] DNA nanotechnology: Nucleic acid nanoparticles can be composed of DNA or RNA. Both share some of the principles in nanoparticle construction and assembly and can be fabricated into similar architectures.^{132,133} However, there are some significant differences between DNA and RNA concerning thermostability, folding principles, ion requirements for assembly, and *in vivo* behavior. DNA nanotechnology utilizes mainly Watson-Crick base-pairing for structure folding and assembly; while RNA also utilizes non-canonical base-pairing to add further structure diversity, stability, motility, and deformability. Both DNA and RNA can produce branched motifs that allowed for the generation of origami through intermolecular interactions.^{133,134} DNA provides similar tunability and programmable design to RNA to produce wire-framed DNA nanoparticles that have been explored as drug delivery vehicles.¹³⁵ The use of DNA aptamers can provide DNA nanoparticles with the needed active targeting of tumor cells and studies have demonstrated the *in vitro* feasibility to deliver gene silencing therapeutics and intercalated chemical drugs (i.e. doxorubicin).¹³⁵⁻¹³⁷ However, DNA architectures have a lower thermodynamic stability compared to RNA and generally require a minimum of 10 mM magnesium ions for nanoparticle folding into a stable structure;^{46,135} while the magnesium concentration *in vivo* is lower than the 10 mM needed. Additionally, DNA nanoparticles must address, through careful design, sensitivity to nucleases as double-stranded DNA is stable since the human body lacks dsDNA degradation enzymes, but single-stranded DNA is sensitive to ssDNase degradation that is present *in vivo*. DNA nanoparticles have successfully conjugated paclitaxel; however, designs were limited to one molecule per DNA strand.^{138,139} This limits the drug loading capacity compared to the 6 conjugated drugs per strand on the RNA 4WJ and would require significantly more complex DNA nanoparticle design to reach a higher number of drug molecules per nanoparticle. Conjugation of multiple chemotherapeutics per oligo would destabilize the DNA nanoparticles much like in the less thermostable RNA 3WJ nanoparticle compared to the RNA 4WJ.³⁹

The 4WJ nanoparticle described in this Protocol relies on base pairing to form its highly stable singular structure, compared to other RNA nanoparticle platforms that assemble from multiple subunits into nano-complexes through intermolecular kissing loops or bulge interactions, such as tectoRNA, and sticky ends.^{66,140} These strategies have generated several RNA nanoparticles into 2D nanostructures, 3D prisms, and nanoarrays.^{59,64,87,90,140-143} RNA nanoparticles of more complex design^{51,57,84,91,144-147} with a size larger than the 4WJ may have the ability to allow for drug conjugation and may increase drug loading over the 4WJ; however, changes in particle size and shape greatly influence biodistribution, toxicity, and immunostimulant properties.^{36,42,48} Further studies of other larger and complex RNA nanoparticles would have to be conducted and evaluate other RNA nanoparticle platforms to optimize anti-tumor to toxicity profiles.

[H2] Advantages and limitations

RNA is an anionic polymer^{21,148-152} that holds two unique properties: Structurally, it is thermostable with high T_m values; while conformationally, it is dynamic^{151,153,154} and deformable.^{150,155} RNA as a biopolymer with unique base-pairing nature and its special charged backbone leads to the following structuring particularities: near-neighbor principles,¹⁵⁶⁻¹⁵⁸ strand-breathing,^{21,159} pseudoknot formation,¹⁶⁰⁻¹⁶² induced-fit,¹⁶³⁻¹⁶⁵ and conformation-capture.¹⁶⁶⁻¹⁶⁸ Thus, RNA is motile, dynamic,^{151,153,154} deformative,^{20,21} elastic,^{20,169,170} and a primitive living material.¹⁷¹⁻¹⁷³ Its anionic nature prevents its nonspecific entry into healthy cells that also have a negatively charged lipid membrane resulting in repulsion by electrostatic charges. These advantages of RNA contribute to their spontaneous and high efficiency tumor targeting, with fast excretion from the body without toxicity. RNA nanoparticles are advantageous for the delivery of therapeutics to solid tumors, as demonstrated across several tumor models including colorectal cancer,^{41,71,174} triple negative breast cancer (TNBC),^{32,33,47} non-small cell lung cancer (NSCLC),³¹ prostate cancer,²⁹ ovarian cancer,^{175,176} glioblastoma,^{69,73} and gastric cancer.⁷⁰ RNA is dynamic, thus it undergoes restructuring events generating motion and wobble.²⁰ When developed into nanoparticles, RNA assembles into defined shapes but retains its dynamic property.^{21,43} It was also shown that RNA nanoparticles are elastic,²⁰ in that they deform under external forces and return to their native structure upon relaxation of forces. These dynamic and deformable properties allow the RNA nano-scaffolds to slip through the actively growing blood capillaries in tumor vasculature. Additionally, these properties result in rapid renal excretion via the 5.5-nm glomerular filter preventing interactions and accumulation in healthy organs.^{20,46} Thus, RNA nanoparticles provide a beneficial biodistribution profile of over 5% of injected dose accumulating in the tumor environment without showing long-term accumulation in healthy organs or pathological change to these tissues.^{37,39,42} The immunogenicity of RNA nanoparticles is shape, size, sequence and stoichiometry dependent.^{49,177} RNA nanoparticles can thus be modified to serve as immune adjuvants⁴⁸ or can be designed as 4WJ that has not induced immune response.⁴² As such these nanoparticles have proven to be a safe delivery vehicle of previously poorly tolerated chemotherapeutics. Additionally, RNA nanoparticles allow for the combination delivery of therapeutics such as chemical drugs with RNAi³⁴ or multiple chemotherapeutics (such as gemcitabine and SN38) to result in synergistic inhibition of tumors (**Fig. 6**).⁴¹

While RNA 4WJ nanoparticles provide numerous benefits and are an ideal drug delivery vehicle, the number of RNA nanoparticles translating into the clinic is currently limited. One of the largest limitations of RNA nanotechnology was the scalability in production and its related cost of synthesis.^{19,21,178,179} The challenge of cost has been resolved by the industrial RNA production at GMP grade. Industrial scaling of RNA production can now produce gram or kilogram-scale batches of RNA complex. Due to the 98.5% efficiency limit of nucleotide incorporation, solid-phase synthesis of RNA is limited to 120 nucleotides in length to ensure the yield of the full-length RNA in the final products. This can limit the design of RNA nanoparticles to maximize drug loading as ~5 nt spacing between drug conjugation sites is required to prevent drug aggregation.³⁹ A possible solution is to produce RNA dendrimers,^{55,180} six-way junctions,³⁴ or larger RNA nanoparticles similar to replacing a large Lego brick with several smaller pieces having the same overall footprint. However, for solid tumor therapy, production of larger RNA complexes will introduce the problem of unfavorable biodistribution due to macrophage engulfment. Currently production of RNA nanoparticles with alkyne modifications cannot be done via *in vitro* transcription using T7 RNA polymerase as 2'-propargyl modified triphosphate nucleotides are not commercially available. However, in the future, these nucleotides could be produced and incorporated into long RNA oligos by mutant T7 RNA polymerases. Currently, each strand comprising the 4WJ is ~40

nt and incorporation of most RNA aptamers still remains within the limitations of solid-phase synthesis.

To improve this technique, enhanced RNA folding models need to be developed. The field of RNA folding is progressing in predicting RNA structures, as shown with the release of AlphaFold 3,¹⁸¹ RNAComposer,^{92,182} and Rosetta and trRosettaRNA^{183,184} for predicting the RNA structure. Finally, the longstanding concern of RNA instability in circulation and susceptibility to RNases has been solved by the use of 2'-nucleotide modifications, such as 2'-Fluorine or 2'-OMethyl.^{46,185,186} Such modifications as well as the 2'-propargyl modification used here, allow for RNA nanoparticles to remain stable in the presence of serum RNases for more than 36 hours.^{39,185}

[H2] Experimental design

Here we discuss the design criteria for generating stable RNA nanoparticles that allow for the conjugation of chemotherapeutics without destabilizing the nanoparticles, preventing drug aggregation, and loading enough drug for strong therapeutic response.

[H3] RNA nanoparticle design

In this Protocol, we describe the conjugation of paclitaxel, camptothecin, and SN38 to our developed 4WJ RNA nanoparticle.³⁹⁻⁴¹ This protocol is highly adaptable, and the experimental design can be varied. In the design of the RNA nanoparticle, one must consider the cancer type being targeted, the chemotherapeutic to be conjugated, the possible combination with RNAi components, and the nanoparticle itself.³⁴ Any RNA nanoparticle can be used, as long as it includes the 2'-propargyl modifications and demonstrates high enough stability, as discussed above. The folding and thermodynamic stability of a nanoparticle can be modeled through computational modeling or experimental testing before following this protocol, to ensure stability above 70 °C.¹⁸⁷⁻¹⁹³ Additionally, due to the variety of modified phosphoramidites this protocol can be adapted to use other desired chemicals for linking chemical drugs to the RNA sugar chain.

[H3] Selection for targeting ligand for specific tumor types

The features of the targeted tumor or cell type are of importance for selecting a targeting ligand. RNA aptamers, DNA aptamers, and chemical ligands have all previously been used on RNA nanoparticles and allow for the targeting of specific receptors on tumor cells.^{29,31-33,41,47,69-71,73,174-176} For example, EGFR has been shown to be over-expressed on several tumors including TNBC^{32,194} or NSCLC^{31,195} and the inclusion of an EGFR binding aptamer has allowed for specific targeting of these tumors by RNA nanoparticles. Prostate specific membrane antigen (PSMA) is over-expressed in prostate cancer subtypes and has become a popular receptor to target due to the high affinity of its RNA aptamer.¹⁹⁶⁻¹⁹⁸ Any tumor type can be targeted by RNA nanoparticles with the vast library of available ligands.¹⁹⁹ The modularity and adaptability of RNA nanoparticles allow for the exchange of targeting ligands to the desired target.

[H3] Selection of chemotherapeutics for conjugation

There is a wide variety of chemotherapeutics and anti-cancer chemical drugs on the market that can be utilized for conjugation into RNA nanoparticles. Many of these can be substituted into the RNA nanoparticle over the described paclitaxel, camptothecin, and SN38. The total number of drug conjugates can be controlled through the number of 2'-propargyl modifications included on each RNA oligo strand. If fewer modifications are included, the total number of chemotherapeutic molecules will decrease per RNA nanoparticle and may result in decreased anti-tumor effects and limit nanoparticle performance. Additionally, the number of 2'-propargyl modified nucleotides can be increased; however, they should be spaced by at least 5

nucleotides. Closer spacing can lead to nanoparticle destabilization and aggregation of the chemical drug. If this occurs, RNA nanoparticles will not remain stable *in vivo* resulting in off target delivery and drug aggregates will not be covalently linked to the RNA nanoparticle which will result in early release during circulation. RNA nanoparticles are highly adaptable, but the design parameters must be carefully tuned to ensure desired results.

[H3] Conjugating additional molecules to RNA nanoparticles

Further, additional chemicals of interest can be conjugated to the RNA nanoparticle, not just chemotherapeutics. To directly follow the protocol here, a chemical must have a hydroxyl (-OH) group that is readily reactive for the addition of the azido hexanoic acid linker through Steglich esterification.¹⁰² However, many additional chemical reactions can be utilized for other chemicals, such as NHS ester chemistry to add an azido linker to a chemical or drug to be conjugated. NHS ester chemistry would allow the conjugation of a drug with a primary amine to click onto an RNA nanoparticle, vastly diversifying the number of chemicals that can be used to modify the RNA oligos. One must take into consideration the release of the chemical or drug from the RNA nanoparticle and its ability to return to its original structure and function. To that end, nucleic acid nanoparticles (DNA and RNA) have utilized several stimuli-responsive linkers to conjugate chemical drugs to the nanoparticles.^{39,139,200,201} Prodrug release has been designed through linkers that are tumor sensitive via changes in pH (i.e. hydrazone or imine), hypoxia (i.e. azobenzene), and reactive oxygen species (ROS) (i.e. thioketal). Additionally, linkers have been designed to be cleaved by tumor associated enzymes such as esters, peptides, and L-γ-glutamylglycine; as well as glutathione (GSH) responsive linkers such as disulfide linkers that are responsive in the extracellular matrixes of tumors. These linkers have been well reviewed by others and can be adapted into RNA nanoparticles through the generation of prodrugs.²⁰²⁻²⁰⁵

[H3] Expertise needed to implement the protocol

The conjugation of chemotherapeutics to RNA nanoparticles combines concepts and procedures from several scientific disciplines, including nucleic acid chemistry, organic chemistry, analytical chemistry, molecular biology, and the testing of the nanoparticles requires cellular biology and animal care skills. With that said, the procedures detailed can be followed by a competent graduate student, postdoctoral researcher, or similar. If the researcher lacks skills or training in an individual part of the protocol, many research institutes provide core facilities that can be used.

[H3] Regulatory approvals

The procedures described involve several health risks that must receive regulatory approvals before completing the experiments. Some institutes have safety considerations for the generation and manipulation of nanoparticle materials. While RNA nanoparticles generally do not fall into a nanoparticle hazard, compared to graphene, heavy metals, or polymer based particles, it is still important to check the required approval status of RNA nanoparticles. Additionally, RNA nanoparticles, depending on the institute, may fall under recombinant nucleic acid safety requirements. RNA nanoparticle sequences do not match any gene in the human body and are fully synthetic, so they are often exempt from these regulations. The testing of the RNA nanoparticles in cell lines will require the Institutional Biosafety Committee (IBC), or similar, review before completing experiments. These procedures have previously been approved by the University of Kentucky and The Ohio State University's IBC and one can expect the approval process to take 1-3 months.

[H1] MATERIALS

[H2] Biological materials

!CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

MDA-MB-231 human breast cancer cells (ATCC, cat. no. HTB-26; RRID:CVCL_0062)

RAW 264.7 mouse macrophage cells (ATCC, cat. no. TIB-71; RRID:CVCL_0493)

[H2] Reagents

CRITICAL: Unless specified, all the reagents can be replaced with other brands, if available.

!CAUTION: All phosphoramidites are moisture sensitive and reactive in water. All phosphoramidites should be freshly dissolved in anhydrous acetonitrile and only be opened in moisture free environment, such as dried argon in a chemical glove box. Dissolved phosphoramidites should be disposed of after 3 days of storage in argon atmosphere.

- **(S)-(+)-Camptothecin (Sigma-Aldrich, cat. no. PHL89593)**
!CAUTION: Camptothecin is toxic if swallowed and may cause genetic defects. Wear gloves and goggles when handling.
- **1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Thermo Scientific, cat. no. 22980)**
!CAUTION: EDC is moisture sensitive. May cause eye and skin irritation. Wear gloves and goggles when handling.
- **2'-tBDSilyl Adenosine (n-bz) CED phosphoramidite (Chemgenes, cat. no. ANP-5671)**
!CAUTION: Moisture sensitive.
- **2'-tBDSilyl Cytidine (n-acetyl) CED phosphoramidite (Chemgenes, cat. no. ANP-6676)**
!CAUTION: Moisture sensitive.
- **2'-tBDSilyl Guanosine (n-ibu) CED phosphoramidite (Chemgenes, cat. no. ANP-5673)**
!CAUTION: Moisture sensitive.
- **2'-tBDSilyl Uridine CED phosphoramidite (Chemgenes, cat. no. ANP-5674)**
!CAUTION: Moisture sensitive.
- **2'-Fluoro-2'-deoxy Cytidine (n-ac) CED phosphoramidite (Chemgenes, cat. no. ANP-9152)**
!CAUTION: Moisture sensitive.
- **2'-Fluoro-2'-deoxy Uridine CED phosphoramidite (Chemgenes, cat. no. ANP-9154)**
!CAUTION: Moisture sensitive.
- **2'-O-propargyl Cytidine (n-bz) CED phosphoramidite (Chemgenes, cat. no. ANP-7752)**
!CAUTION: All phosphoramidites are moisture sensitive.
- **2'-O-propargyl Uridine CED phosphoramidite (Chemgenes, cat. no. ANP-7754)**
!CAUTION: Moisture sensitive.
- **3% TCA in Dichloromethane (Fisher Scientific, cat. no. 40-4140-62)**
!CAUTION: TCA in Dichloromethane causes serious eye damage, is suspected of causing cancer, and causes skin irritation. Wear gloves and goggles and dispense in a vented hood.
- **4-(dimethylamino)pyridine (Sigma-Aldrich, cat. no. 107700)**

!CAUTION: 4-(dimethylamino)pyridine causes serious acute toxicity and damage to organs through oral, inhalation, and skin contact. Wear gloves, goggles, and dispense it in a vented hood.

- **40% aqueous MethylAmine (Sigma, cat. no. 426466-100ML)**

!CAUTION: MethylAmine solution is highly flammable and harmful through skin contact or if swallowed or inhaled. Wear gloves, goggles, and dispense in a vented hood.

- **5'-Hexynyl Phosphoramidite (Glen Research, cat. no. 10-1908-02E)**

- **5'-Amino-Modifier (Glen Research, cat. no. 10-1906-02E)**

- **6-azido-hexanoic acid (Chem-IMPEX, cat. no. 29363)**

- **Acetic acid, glacial (HAc, Fisher, cat. no. A38C-212)**

!CAUTION: HAc is a weak acid; it is corrosive and is a skin, eye, and respiratory tract irritant. Wear gloves, goggles, and dispense in a vented hood.

- **Acetonitrile (HPLC grade) (ACN, Fisher, cat. no. A998SK-4)**

!CAUTION: Acetonitrile (ACN) is highly flammable, can cause serious eye irritation and is harmful if swallowed. Wear gloves and goggles.

- **Acetonitrile anhydrous (Fisher Scientific, cat. no. BP1170-4)**

!CAUTION: Acetonitrile is highly flammable, can cause serious eye irritation and is harmful if swallowed. Wear gloves and goggles.

- **Acrylamide, 99+%, electrophoresis grade (Acros Organics, cat. no. 164850025)**

!CAUTION: Acrylamide may cause cancer and heritable genetic damage. Harmful by inhalation and in contact with skin. Toxic if swallowed. Wear gloves and a mask.

- **Acrylamide/Bis 40% (wt/vol) solution, acrylamide/bis-acrylamide ratio 29:1 (Fisher, cat. no. BP1408-1)**

!CAUTION: Acrylamide is highly toxic and is a carcinogen. Wear gloves and goggles.

- **Activator, 0.25M 5-Ethylthio-1H-Tetrazole (ETT) in Anhydrous Acetonitrile (Sigma, cat. no. L030000-6X450ML)**

!CAUTION: Causes severe skin burns and eye damage and may cause respiratory irritation. Wear gloves and goggles.

- **Alexa Fluor 488 phalloidin (ThermoFisher Scientific, cat. no. A12379)**

!CAUTION: Alexa Fluor 488 phalloidin can cause acute toxicity if swallowed, by contact with skin, or inhaling. Wear gloves, goggles, and dispense in a vented hood.

- **Ammonium acetate (NH₄OAc, J.T. Baker, cat. no. 0596-01)**

- **Ammonium carbonate (Sigma, cat. no. 207861-25G)**

- **Ammonium hydroxide solution (Sigma, cat. no. 05003-1L)**

!CAUTION: Ammonium hydroxide causes severe skin burns, eye damage, and respiratory irritation. Wear gloves and goggles.

- **Ammonium persulfate (APS, Fisher, cat. no. BP179-100)**

!CAUTION: APS is harmful if swallowed, causes skin irritation, and serious eye irritation. Wear gloves, goggles, and dispense in a vented hood.

- **Bis-Acrylamide (Fisher, cat. no. BP171-100)**

!CAUTION: Bis-Acrylamide causes eye, skin and respiratory tract irritation. Harmful if inhaled, swallowed, or absorbed through skin. May cause central nervous system effects. Wear gloves, goggles, and mask.

- **Boric acid (Fisher, cat. no. A74-500)**

!CAUTION: Boric acid may damage fertility and the fetus during pregnancy.

- **Bromophenol blue (Sigma, cat. no. B-8026)**
!CAUTION: Bromophenol blue is a skin and respiratory tract irritant.
- **Capping reagent A - THF/Lutidine/Acetic Anhydride (8:1:1) (Bioautomation, cat. no. BIO221/0450)**
- **Capping reagent B - 16% n-Methylimidazole in THF (Bioautomation, cat. no. BIO345/0450)**
- **CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, cat. no. G4000)**
- **Clorox regular bleach (VWR, cat. no. 37001-058)**
!CAUTION: Bleach is corrosive, and can cause severe irritation or damage to eyes and skin. It is harmful if swallowed.
- **Copper(I) Bromide (CuBr) (Sigma-Aldrich, cat. no. 212865)**
!CAUTION: Causes skin irritation, eye damage, and harmful if swallowed. Wear gloves and goggles.
- **Cystamine dihydrochloride, 97% (Acros Organics, cat. no. 111770250)**
!CAUTION: Cystamine causes eye, skin, and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Wear gloves, mask and goggles.
- **Deuterated Chloroform (Cambridge Isotope Laboratories Inc., cat. no. DLM-7-10X0.6)**
!CAUTION: Causes skin irritation and eye damage. Wear gloves and goggles.
- **Dichloromethane anhydrous (DMC, Fisher Scientific, cat. no. AC348460025)**
!CAUTION: Causes skin irritation and eye damage. Wear gloves and goggles.
- **Diethyl pyrocarbonate (DEPC, cat. no. Sigma, D-5758)**
!CAUTION: Causes acute oral toxicity. Wear gloves.
- **4-Dimethylaminopyridine (DMAP, ThermoFisher Scientific, cat. no. 148270250)**
- **Dimethyl sulfoxide (DMSO, Acros Organics, cat. no. 348445000)**
!CAUTION: Avoid contact with skin and eyes.
- **DMEM (ThermoFisher Scientific, cat. no. 11965092)**
- **DMEM/F-12 medium (ThermoFisher Scientific, cat. no. 11320033)**
- **EDTA disodium salt, dihydrate (Sigma, cat. no. E-5134)**
!CAUTION: EDTA is a skin, eye, and respiratory tract irritant.
- **Ethanol, 100% (Pharmco-AAPER, cat. no. 111000200)**
!CAUTION: Ethanol is a flammable liquid.
- **Ethidium bromide (EtBr, Fisher, cat. no. P1302-10)**
!CAUTION: EtBr is a strong carcinogen; it is a skin, eye, and respiratory tract irritant. Wear gloves and dispense in a chemical hood.
- **Ethyl Acetate (JT Baker, cat. no. 9280-03)**
!CAUTION: Causes skin irritation and eye damage. Wear gloves and goggles.
- **Ethylenediamine dihydrochloride (Thermo Scientific, cat. no. 23031)**
!CAUTION: Ethylenediamine may cause eye, skin and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Wear gloves, mask, and goggles.
- **exACT gene 50bp mini DNA ladder (Fisher, cat. no. BP2570100)**
- **Fetal bovine serum, heat inactivated (FBS, S cat. no. igma, F4135)**
- **FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, cat. no. 556570)**
- **Formamide (Fisher, cat. no. F84-1)**

!CAUTION: Formamide is harmful if swallowed, inhaled or absorbed through the skin. Cause eye and skin irritation. May cause respiratory tract irritation, central nervous system effect, and liver damage. Wear gloves.

- **Glen Gel-Pak™ 2.5 Desalting Column (Glen Research, cat. no. 61-5025-25)**
- **Hydrochloric acid (HCl; Fisher, cat. no. A144-212)**

!CAUTION: HCl is a strong acid; it is very corrosive and is a skin, eye, and respiratory tract irritant. HCl can cause severe burns. Wear gloves and goggles, and dispense in a vented hood.
- **Magnesium chloride hexahydrate (MgCl₂, Fisher, cat. no. M33-500)**
- **Methanol (Fisher, cat. no. A412-4)**

!CAUTION: Methanol is a dangerous, poisonous, and flammable liquid and vapor. Harmful if inhaled. May be fatal or cause blindness if swallowed. May cause eye and skin irritation. May cause central nervous system depression, kidney damage, reproductive and fetal effects. Wear gloves, goggles, and dispense in a vented hood.
- **Milli-Q water, 18.2 MΩ cm⁻¹ resistivity**
- **Molecular Trap (Bioautomation, cat. no. BG7-0013-1)**
- **Mouse ELISA MAX Deluxe sets (BioLegend, cat. no. 433404)**
- **Mouse IFN-α ELISA Kit (R&D Systems, cat. no. MFNAS0)**
- **n-Hexane (C₆H₁₄, Mallinckrodt Chemicals, cat. no. 5189-08)**

!CAUTION: Flammable liquid, causes skin irritation and eye damage. Wear gloves and goggles.
- **N,N'-dicyclohexycarbodiimide (Acros Organics, cat. no. AC113901000)**
- **Opti-MEM Reduced Serum Medium (Thermo Fisher, cat. no. 31985062)**
- **Oxidizer, 0.02M Iodine in Tetrahydrofuran/Pyridine/Water (70:20:10) (Sigma, cat. no. L260045-06)**

!CAUTION: Highly flammable liquid and vapor, harmful if swallowed or in contact with skin, causes skin irritation and serious eye irritation. Wear gloves, goggles, and dispense in a vented hood.
- **Paclitaxel (ThermoFisher Scientific, cat. no. J62734.ME)**

!CAUTION: Paclitaxel causes skin irritation, serious eye damage, and may cause respiratory irritation. Wear gloves, goggles, and dispense in a vented hood.
- **Paraformaldehyde (4% wt/vol; Biotium, cat. no. 22023)**

!CAUTION: Flammable solid, harmful if swallowed or in contact with skin, causes skin irritation and serious eye irritation. Wear gloves, goggles, and dispense in a vented hood.
- **Penicillin-streptomycin (PS, Gibco by Life Technologies, cat. no. 15070-063) 0.25% (wt/vol)**
- **Phosphate Buffered Saline (PBS, pH 7.4; Thermo Fisher, cat. no. 10010023)**
- **Prolong® gold antifade reagent with DAPI (Life Technologies, cat. no. P36935)**
- **RediSep® Empty Disposable Sample Load Cartridges - 65 Gram Hold (Teledyne ISCO, cat. no. 69-3873-225)**
- **RediSep® Silver Silica Gel Disposable Flash Columns - 12 Gram (Teledyne ISCO, cat. no. 69-2203-312)**
- **Silica gel 60 – 200 mesh (SiO₂, Milipore Sigma, cat. no. SX0143U-1)**

!CAUTION: Silica gel causes eye damage, and may cause respiratory irritation. Wear gloves, goggles, and dispense it in a vented hood.

- **SN38 (7-Ethyl-10-hydroxycamptothecin; Sigma, cat. no. H0165)**
!CAUTION: SN-38 is harmful if swallowed causing acute toxicity, reproductive toxicity, and specific target organ toxicity. Wear gloves and goggles.
- **Sodium acetate trihydrate (NaOAc, Fisher, cat. no. S209-3)**
- **Sodium Bicarbonate (NaHCO₃, Milipore Sigma, cat. no. S5761-500G)**
- **Sodium chloride (NaCl, Fisher, cat. no. S271-500)**
- **Sodium dodecyl sulfate (SDS, Fisher, S529-500)**
!CAUTION: SDS is an eye and respiratory tract irritant.
- **Sodium hydroxide (NaOH, Fisher, cat. no. S318-3)**
!CAUTION: NaOH is corrosive and can cause chemical burns. Wear gloves, lab coat, and eye protection when handling the material or its solutions. Dissolution of NaOH is highly exothermic, and the resulting heat may cause heat burns or ignite flammables. It also produces heat when reacted with acids.
- **Sodium Sulfate Anhydrous (Na₂SO₄, Macron Fine Chemicals, cat. no. MFCD00003504)**
- **Sucrose (Fisher, cat. no. S5-3)**
- **Sulfuric Acid fuming (H₂SO₄, Milipore Sigma, cat. no. 435597-500G)**
!CAUTION: H₂SO₄ is corrosive and can cause chemical burns. Wear gloves, lab coat, and eye protection when handling the material or its solutions. Dissolution of H₂SO₄ is highly exothermic, and the resulting heat may cause heat burns or ignite flammables.
- **SYBR Green II dye (ThermoFisher Scientific, cat. no. S7564)**
- **TEMED, electrophoresis grade (Fisher, cat. no. BP150-20)**
!CAUTION: TEMED is a skin and respiratory tract irritant.
- **Tert-butanol (Sigma, cat. no. 360538)**
!CAUTION: Tert-butanol is a highly flammable liquid and vapor, causes serious eye irritation, is harmful if inhaled, and may cause respiratory irritation. Wear gloves and goggles.
- **Triethylamine Acetate (TEAA), 2.0 M, pH=7 (Glen Research, cat. no. 60-4110-60)**
!CAUTION: TEAA causes serious skin and eye irritation and may cause respiratory irritation. Wear gloves and goggles, and dispense in a vented hood.
- **Triethylamine trihydrofluoride (Fisher Scientific, cat. no. AAL1441706)**
- **Tris base (Fisher, BP-152-5)**
- **Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (Sigma-Aldrich, cat. no. 678937)**
- **Triton X-100 (Sigma-Aldrich, cat. no. 93443)**
- **Trypsin–EDTA (1×) (Gibco by Life Technologies, cat. no. 15140-122)**
- **Universal Support Column, DMT off (N-iPr), 1000 Å, 1 µmol, Luer (Bioautomation, cat. no. MLX1-3500-1)**
- **Urea, for molecular biology, DNase-free, RNase-free, and protease-free (Acros, cat. no. 327380050)**
- **Water (HPLC grade) (Fisher, cat. no. W5-4)**
- **Xylene cyanol (Sigma, cat. no. X-4126)**
!CAUTION: Xylene cyanol is a skin and respiratory tract irritant.

[H2] EQUIPMENT

- Agilent 1260 infinity HPLC system
- Amicon® Ultra Centrifugal Filter, 10 kDa MWCO (Sigma, cat. no. UFC501008)
- Avance 300MHz NMR I (Bruker)
- Biosafety cabinet (Thermo Scientific, model 1307)
- Biosset ASM-800 synthesizer
- Branson 1510 benchtop sonicator
- CombiFlash – NextGen 300+ (Teledyne ISCO)
- Eppendorf centrifuge 5424 (Eppendorf)
- Flexible TLC plates (Selecto Scientific, cat no. 11078)
- Forma series II water jacket CO₂ incubator (Thermo Scientific)
- Freezer (–80°C and –20°C) and 4°C refrigerator
- FV3000 Confocal Microscope (Olympus)
- Handheld Mineralight® lamp (UVP, model UVGL-25)
- LSRII Flow cytometer (Bioscience)Malvern zetasizer
- Microfluoro 1 96-well white Microtiter plates (Thermo Scientific, cat no. 14-245-194A)
- Milli-Q water purification system
- NanoDrop 2000 spectrophotometer (Thermo Scientific)
- Olympus FV3000 confocal microscope (Olympus Corp.)
- pH meter (Fisher, Dual channel pH/ion meter AR25)
- PCR machine (Eppendorf Mastercycler Gradient, model 5331)
- Real-time PCR detection system (Roche LightCycler 480 real-time PCR System)
- PAGE gel electrophoresis system (Hoefer, SE250 mighty small II for 8 x 7 cm gel)
- PLRP-S 4.6 × 250 mm 300 Å column (Agilent Technologies)
- TGGE system (Biometra, model 024-000)
- Optical microscope (Motic company, cat. no. SFC-11)
- SAVANT DNA120 Speed Vac concentrator (Thermo Electron Corporation)
- Synergy 4 microplate reader (Bio-Tek)
- Typhoon FLA 7000 imaging system (GE Healthcare)
- Waters Acquity UPLC and ESI-MS with PDA and QDA Detectors (Waters Corporation)
- *Mfold* (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>)
- *FlowJo* (<https://www.flowjo.com/solutions/flowjo/downloads>)
- *ImageJ* (<http://rsbweb.nih.gov/ij/download.html>), free download and installation. User instruction is also available online (<http://rsbweb.nih.gov/ij/>).
- *MassLynx* (https://www.waters.com/waters/en_US/MassLynx-Mass-Spectrometry-Software/nav.htm?locale=-&cid=513662)
- *Mestrelab Nova* (<https://mestrelab.com/>)

[H2] REAGENT SETUP

▲**CRITICAL:** Prepare all reagents and perform all experiments using pure water (Milli-Q, 18.2 MΩ cm⁻¹ resistivity). Use DEPC-treated water for experiments involving RNA.

!CAUTION: Wear gloves at all times.

- Click Solvent: Three parts DMSO, one part tert-butanol (3 ml DMSO and 1 ml tert-butanol). Should be freshly prepared to limit solvent becoming wet.
- CuBr Stock Solution: Freshly prepared 0.1 M copper bromide in click solvent (1 mg CuBr dissolved in 70 μ l click solvent).
- TBTA Stock Solution: 0.1 M solution of TBTA in click solvent (54 mg TBTA dissolved in 1 ml click solvent). Can be stored at -20°C for 1 year.
- Ascorbic acid Stock Solution: 0.1 M solution of ascorbic acid in click solvent (10.0 mg ascorbic acid dissolved in 570 μ l click solvent). Can be stored at -20°C for 1 year.
- Click Solution: Add one volume of CuBr stock solution to two volumes of TBTA stock solution and 50 volumes of ascorbic acid to a final concentration of CuBr of 1.88 μ M. Solution should be freshly mixed due to oxidation of Cu¹⁺ to Cu²⁺. Ascorbic acid will convert Cu²⁺ back to Cu¹⁺ during the reaction.
- 0.05% (vol/vol) DEPC treated H₂O: Add 0.05 ml of DEPC to 99.5 ml of pure water and shake the solution vigorously. Incubate at 37°C overnight, and then autoclave the solution to remove DEPC. This reagent can be stored in room temperature (RT, 23°C) for 1 year.
- 0.5 M EDTA, pH 8: Dissolve EDTA in pure water. Stir vigorously and adjust pH to 8 with NaOH. This reagent can be stored at RT for 1 year.
- TES buffer: 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA. This buffer can be stored at RT for 1 year.
- Tris-Borate EDTA (TBE) buffer, 1×: TBE buffer (1×) contains 89 mM Tris base, 200 mM boric acid, and 2 mM EDTA. This buffer can be stored at RT for 1 year.
- Tris-Borate Magnesium (TBM) buffer, 1×: TBM buffer (1×) contains 89 mM Tris base, 200 mM boric acid, and 5 mM MgCl₂. This buffer can be stored at RT for 1 year.
- 3 M NaOAc, pH 6.5: Dissolve NaOAc in pure water. Adjust pH to 6.5 with HAc. After autoclaving, this buffer can be stored at RT for 1 year.
- 2 M MgCl₂: This buffer can be stored at RT for 1 year.
- Tris-EDTA Saline (TES) buffer, 1×: TES buffer (1×) contains 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 2 mM MgCl₂. This buffer can be stored at RT for 1 year.
- Urea denaturing PAGE gel, 10–15% (wt/vol): Combine 10–15% (wt/vol; 37.5:1) acrylamide, 8 M urea, 10% (wt/vol) APS and TEMED. The gel should be freshly prepared and filtered to remove excess Urea.
- Native PAGE gel (TBM or TBE), 10–15% (wt/vol): Combine 10–15% (wt/vol; 37.5:1) acrylamide, 1× Tris-borate (TB) buffer (pH 7.8), 10 mM MgCl₂ for TBM (or 2 mM EDTA for TBE), 10% (wt/vol) APS and TEMED. The gel should be freshly prepared.
- RNA elution buffer, 1×: RNA elution buffer (1×) contains 0.5 M NH₄OAc, 10 mM EDTA, 0.1% (wt/vol) SDS in 0.05% (vol/vol) DEPC treated water. After autoclaving, this buffer can be stored at RT for 6 months.
- 6× loading buffer: 6× loading buffer contains 40% (wt/vol) sucrose, 0.1% (wt/vol) xylene cyanol FF, and 0.1% (wt/vol) bromophenol blue. This buffer can be stored at -20 °C for 1 year.
- 2× TBE loading buffer: 2× TBE loading buffer contains 95% (vol/vol) formamide, 18 mM EDTA, 0.025% (wt/vol) SDS, 0.025% (wt/vol) bromophenol blue, and 0.025% (wt/vol) xylene cyanol. This buffer can be stored at RT for 1 year.
- 10 % (wt/vol) APS: 10% (wt/vol) APS should be freshly made.

- 0.05 M phosphoramidite solution: Solid phosphoramidite (2'-tBDSilyl Adenosine (n-bz) CED phosphoramidite, 2'-tBDSilyl Cytidine (n-acetyl) CED phosphoramidite, 2'-tBDSilyl Guanosine (n-ibu) CED phosphoramidite, 2'-tBDSilyl Uridine CED phosphoramidite, 2'-Fluoro-2'-deoxy Cytidine (n-ac) CED phosphoramidite, 2'-Fluoro-2'-deoxy Uridine CED phosphoramidite, 2'-O-propargyl Cytidine (n-bz) CED phosphoramidite, 2'-O-propargyl Uridine CED phosphoramidite, 5'-Hexynyl Phosphoramidite, or 5'-Amino-Modifier) is mixed with acetonitrile anhydrous to reach a final concentration of 0.05 M of each phosphoramidite. **CRITICAL** The solution should be freshly prepared within an argon atmosphere to prevent water contamination.
- 1.5 M Ammonium carbonate solution: Ammonium carbonate is mixed with water to reach a final concentration of 1.5 M. The solution can be stored at 4 °C in a sealed container for 1 year. Storage at RT may result in decomposition, thus affecting the solution's effectiveness.
- 0.1M TEAA in water: 100 ml of 2.0 M TEAA is mixed with Water (HPLC grade) into a final volume of 2 L. This solution can be stored at RT for 1 year.
- 0.1M TEAA in 75% (vol/vol) Acetonitrile/H₂O: 100 ml of 2.0 M TEAA is mixed with 400 ml of Water (HPLC grade), then Acetonitrile (HPLC grade) is added and fixed at the final volume of 2 L. This solution can be stored at RT for 1 year.

[H2] EQUIPMENT SETUP

[H3] Biosset ASM-800 synthesizer

The procedures listed in Box 1 outline the manufacturer's instructions for operating the oligo-synthesizer.

[b1] Box 1. Operating the ASM-800 oligo synthesizer

▲CRITICAL: Phosphoramidite chemistry is highly sensitive to water, and water contamination greatly reduces reaction efficiency and therefore the yield of RNA synthesized. Ensure all solutions and chemicals used on the oligo-synthesizer are dry and not in the presence of air or wet gas.

[bH1] Open the Synthesizer Software:

1. Launch the Oligo 800 synthesizer software on a computer connected to the equipment.

Pressurize the System:

2. In the Oligo 800 Software window, navigate to "Auto Press" and click "Start" to pressurize the system with argon gas. If the gas cylinder is changed, re-pressurize using "Auto Press".

[bH1] Manual Mode Activation:

3. After pressurizing the synthesizer system, select "Manual" in the Oligo 800 Software menu to display a system diagram.

[bH1] Column Flushing with Acetonitrile:

4. Flush the columns (W1a, W1b, and W2) with acetonitrile. In the "Manual" window, select the columns, set the volume to 1000 µL and the flow rate to 100 µL/sec, and initiate the flow. Repeat thrice to ensure a total of 3 mL of acetonitrile passes through

the columns. During flow, monitor the syringes for bubbles and air intake, ensuring all bottles are sealed tightly.

5. If air is seen in the syringes, check and tighten each attached bottle and repeat the above procedures.

[bH1] Inputting the RNA Sequence to be Synthesized:

6. In the Oligo 800 Software, click “Sequence” and enter your sequence in capital letters or number according to the installed and desired phosphoramidite bottle from 5’ to 3’. For universal columns, input the entire sequence; if a specialty column is used, the first nucleotide may be included on the column. Save the inputted sequence by clicking the “save” button.

[bH1] Mounting the Controlled Pore Glass (CPG) Support Columns:

7. Fit the CPG support columns tightly onto the instrument, ensuring there are no gaps between the metal portion and the column frits.

[bH1] Loading Sequences and Starting Synthesis:

8. In the Oligo 800 Software, click “Run”, delete any previous sequences by selecting the leftmost button on the first line and clicking “delete all”. Load your sequences to the corresponding column locations.

[bH1] Loading the Synthesis Program:

9. In the “Run” window, navigate to “File” and select “Open Program”. Delete any pre-loaded programs and select “200 nm RNA” for RNA synthesis.

[bH1] Final Deblocking Protocol Selection:

10. Ensure the “Dpr” checkbox is checked to pause the synthesis at the final deblocking step in order to observe the final deblocking efficacy and guarantee the quality of synthesis strands.

[bH1] Initiating and finishing the synthesis:

11. Click the start button to begin synthesis. Upon completion, when the program pauses at the final deblocking step, select “Continue” to finish the synthesis.

[bH1] Drying the Columns:

12. In the “Manual” window, select “gas” and then the relevant column numbers, followed by “waste”. Set the time to “100” and click start. Repeat this thrice to dry the CPG thoroughly.

[bH1] Removing the Columns:

13. Carefully disconnect the columns from the synthesizer, keeping the top connector on to avoid losing the column beads and RNA. Optionally, remove a blank column to release residual argon pressure. Place the support column in a 2 mL centrifuge tube.

[bH1] Final Steps:

14. Reattach the blank column to the instrument, then transfer the CPG beads from the support column to the centrifuge tube by pushing them out.

[H2] Agilent 1260 Infinity Quaternary LC

See Box 2 for operating instructions.

[b2] Box 2. Operating the Agilent 1260 Infinity Quaternary LC

[bH1] Startup Procedure

[bH2] Column Installation:

1. Connect the PLRP-S 4.6 × 250 mm 300 Å column to the HPLC system, following manufacturer's instructions.

[bH2] Solvent Attachment:

2. Degas a 1 L bottle of each of the following solvent using a Branson 1510 benchtop sonicator for 30 min. Attach the following buffers to the corresponding tubing connected to the HPLC pump following manufacturer's instructions.

Solvent A: 0.1 M TEAA in HPLC water

Solvent B: 0.1 M TEAA in 75% HPLC ACN

Solvent C: 100% HPLC grade ACN

Solvent D: 100% HPLC grade water

[bH2] Software Initialization:

3. Open the OpenLab (online) software.

[bH2] Method Creation:

4. Select "New method from the instrument" to enter the software interface.

[bH2] Mobile Phase Composition:

5. Right-click on the "Quat. Pump" block and select "Method".
6. Adjust the solvents to 95% C and 5% D (vol/vol) in the pop-up window.
7. Click "Apply" to execute the change.

[bH2] Column Temperature Adjustment:

8. Right-click on the "Column Comp." block.
9. Input the desired temperature for both left and right sides.

[bH2] Signal Acquisition:

10. Set up signal acquisition at 260 nm in the "MWD" module for maximal UV absorption of RNA.

[bH2] Initial Run:

11. Click the green "On" button to start the run with 95% Solvent C and 5% Solvent D (vol/vol) at flowrate of 1.0 mL/min for 30 minutes to allow for equalization of the column.

[bH2] Mobile Phase Change:

12. Change the mobile phase to 50% Solvent A and 50% Solvent B (vol/vol) at 1.0 mL/min for 5 minutes.

[bH2] Column Conditioning:

13. Condition the column with 95% Solvent A and 5% Solvent B (vol/vol) at 1.0 mL/min for 30 minutes.
14. The system is now conditioned and ready for RNA loading for purification.

[bH1] Shutdown Procedure

[bH2] Column Washing:

1. Wash the column with 95% Solvent C and 5% Solvent D (vol/vol) at 1.0 mL/min for at least 30 minutes.
2. Heat the column to 80°C during washing.

[bH2] Baseline Signal:

3. Continue washing until the UV absorbance signal in the online plot reaches baseline.

[bH2] Temperature Adjustment and Flushing:

4. Reduce the column temperature to 25°C.
5. Flush the column using 50% Solvent C and 50% Solvent D (vol/vol).

CRITICAL STEP: Ensure the mobile phase contains at least 1% Solvent C and never use 100% Solvent D (vol/vol).

[bH2] System Shutdown:

6. Power off the HPLC by clicking the red “Off” button in the software.

CombiFlash – NextGen 300+

See Box 3 for instructions.

[bH3] Box 3. Operating the CombiFlash – NextGen 300+

[bH1] Solvent Attachment:

1. Degas a 500 mL bottle of each of the following HPLC grade solvent using a Branson 1510 benchtop sonicator for 30 min. Attach each solvent to the corresponding CombiFlash – NextGen 300+ tubing connected following manufacturer’s instructions.

Solvent A: n-Hexane

Solvent B: Ethyl Acetate

Solvent C: Dichloromethane

Solvent D: Methanol

[bH1] Software Initialization:

2. Open the embedded software within CombiFlash – NextGen 300+. Click on “Reset”

[bH1] Mobile Phase Prime:

3. Click on “Tools” then “Autoprime”
4. Prime with Ethyl Acetate first, and then n-Hexane
5. Click “Play” to prime the system

[bH1] Setting up column and system:

6. Pour dried silica gel provided with sample into solid loading cartridge, put frit onto the top, and pack the cartridge according to manufacturer’s suggestions.

!CAUTION: This step should be completed in a chemical fume hood to avoid inhalation of silica gel.

7. Place solid loading cartridge onto the CombiFlash and connect pipeline with the cartridge according to manufacturer’s suggestions.
8. Place the Silica Gel Disposable Flash Columns onto the CombiFlash. Choose the column type according to manufacturer’s suggestions.
9. In the software, go to “Tools” – “Manual Control” – “Flow Path” – “Through Column and Flow Cell”. Prime with the system with solvent A.
10. Place the tube rack with empty tubes into the CombiFlash.

[bH1] Program set up:

11. In the software, go to method editor.
12. Set up the suggested flow rate and equilibrium volume according to the recommendation on the silica gel column.
13. Collect all fractions by clicking “Peak Collection” – “All”.
14. Select 254 nm, 280 nm, and all wavelength in the “Peak Detection Section”.
15. Set running protocol according to the compounds. For SN38-N₃, the product will wash off at 13% hexane and 87% Ethyl acetate (vol/vol).
 - ▲ **CRITICAL STEP:** Equilibrate the column using 100% *n*-hexane. Avoid using a high percentage of ethyl acetate during equilibration, as it may cause cracking of the silica column. Gradually increase the ethyl acetate fraction during the elution process
16. Save the changes and go back to the main menu in the software.

[bH1] Run and finish:

17. In the software select “Start” on the main menu.
18. Select the start rack and start tube.
19. Select “Solid” under the sample loading option.
20. Disconnect the cartridge and column from the system after finishing.

[H1] PROCEDURE**[H2] Synthesis of 4WJ component strand oligos. TIMING: ~1 wk.**

▲ **CRITICAL:** Phosphoramidite chemistry is highly sensitive to water, and water contamination greatly reduces reaction efficiency and therefore the yield of RNA synthesized. Ensure all solutions and chemicals used on the oligo-synthesizer are dry and not in the presence of wet air or gas.

1. Setup Biosset ASM-800 synthesizer following manufacturer’s procedures and the instructions in Box 1. Prepare synthesizer column onto oligosynthesizer by attaching the Universal Support Column to the synthesizer. Once attached flush the column with ACN three times following Box 1.
2. Input the sequences for 4WJ_A-6 Alk, 4WJ_B-6 Alk, 4WJ_C-6 Alk-EGFR, 4WJ_D-6 Alk, or 4WJ_D-5’ Amine found in **Table 1** or desired nanoparticle sequence. The synthesizer can handle up to eight different sequences simultaneously due to its eight column locations and channels; therefore each strand can be synthesized at once.
3. Initiate RNA synthesis by clicking start to begin the loaded protocol with the following steps. These steps are automated by the synthesizer and do not require user input.
 - i. Addition Step: Add both the acidic catalyst 5-Ethylthio-1H-Tetrazole (ETT) and phosphoramidite to the CPG column. Use a molar ratio of CPG seed to phosphoramidite of 1:20, and ratio of phosphoramidite to 5-Ethylthio-1H-Tetrazole of 1:5. Follow the coupling time recommended by the phosphoramidite provider.
 - ii. Oxidation Step: Post-addition, wash the CPG column with ACN three times. Then add 200 µL of Oxidizer to the column and let it react for 2 minutes.

- iii. Capping Step: After oxidation, wash the CPG column with ACN three times. Add equal amounts of Capping Reagent A and Capping Reagent B, totaling 200 μ L each, and allow the reaction to proceed for 2 minutes.
 - iv. Deprotection Step: Following the capping step, wash the CPG column with ACN three times, then with DCM once. Add 600 μ L of 3% TCA in DCM (vol/vol) as the deprotecting reagent, allowing it to react for 1 minute.
 - v. Repeat Steps: Repeat steps iii through v until the final base is added and deprotected.
4. Following synthesis completion and the final deprotection step, dry the columns using argon gas from the instrument. In the Oligo 800 Software, go to the "Manual" window, select "gas," choose the column numbers, and then "waste." Set the time to "100" and click start. Repeat three times to ensure complete drying.
 - **PAUSE POINT**: RNA oligo synthesis can be paused prior to final deprotection and synthesis. Columns can be stored at -20°C until further use in the short term (6 months).
 5. Remove the column from the synthesizer and transfer the CPG beads into a 2 mL centrifuge tube following the instructions in Box 1.
 6. Add 1.5 mL of AMA solution (1:1 ratio of ammonium hydroxide and methylamine) to the centrifuge tube containing the CPG beads to cleave synthesized RNA from the beads and carry out final base deprotection of base protecting groups of the RNA. Incubate at room temperature for 2 hours. Use the provided metal tube holder to manage the pressure and keep tube lids closed. After incubation, cool the tubes on ice for 10 minutes prior to opening them in a chemical fume hood to release any pressure.
 7. Centrifuge RNA containing tubes using a benchtop mini centrifuge at 2000 g for 30 seconds to spin down the CPG beads, then decant the supernatant containing RNA into a new 1.5 mL centrifuge tube.
 - **PAUSE POINT**: RNA oligo processing can be paused prior to drying and solution can be stored at -20°C until further use in the short term (6 months).
 8. Dry the mixture to remove all solvent using a speed vacuum concentrator overnight.
 9. After drying, resuspend the RNA in 250 μ L of DMSO. Heat to 65°C for ~5 minutes if necessary to fully dissolve the RNA.
 10. Add 250 μ L of TEA.3HF to the RNA/DMSO mixture and incubate at 65°C for 2.5 hours using a metal tube holder to deprotect the 2' location of the RNA ribose. Manage the pressure buildup during heating. Post-incubation, cool the tubes on ice for 10 minutes. Finally, transfer the solution into a new 15 mL centrifuge tube.
 - ▲ **CRITICAL STEP** Wear gloves when handling TEA.3HF.
 11. To neutralize the solution, quench the acid (TEA.3HF) with 1.5 mL of 1.5 M ammonium carbonate. Add the ammonium carbonate slowly and with caution to manage the bubbling reaction.
 12. To desalt and remove early aborted strands, prepare the Glen Gel-Pak™ 2.5 Desalting Column by flushing it with 50 mL of water. Load RNA solution from the centrifuge tube onto the Glen Gel-Pak™ 2.5 Desalting Column. Allow approximately 2 mL of liquid to pass through, then add 1 mL of water at a time and collect five fractions. Fractions 2 to 4 are expected to contain the desalted RNA.

13. Combine the fractions for the further usage and verify RNA concentrations using a Nanodrop or similar UV spectrophotometer. RNA oligos can be stored at -20°C for short term (6 month) or -80°C for long term (>1 year) storage.

[H2] Chemical synthesis of chemotherapeutic prodrug. TIMING: ~1 wk.

14. Add 0.234 mmol (1 equiv.) of 6-azidohexanoic acid, 0.046 mmol (0.2 equiv.) of DMAP, and 0.468 mmol (2.0 equiv.) of chemotherapeutic (paclitaxel, SN-38, or camptothecin) into a dry round bottom flask filled with inert atmosphere gas (i.e. nitrogen or argon gas). Dissolve in 4 ml of anhydrous dichloromethane and mix constantly with a stir bar for 5 minutes at 0°C (in an ice bath).

▲**CRITICAL STEP:** Esterification of the chemical drugs requires a dry environment. The presence of water or humidity could critically affect the yield of prodrug.

15. In another dry round bottom flask filled with inert atmosphere gas (i.e. nitrogen or argon gas), add 0.234 mmol (1 equiv.) of DCC (N,N'-Dicyclohexylcarbodiimide) dissolved in 1 ml of anhydrous dichloromethane and stir reaction for 10 minutes or until all DCC dissolve.

16. Add DCC solution (Step 15) to the reaction mixture (Step 14) dropwise for 5 minutes and continue stirring the reaction for at least 12 hours in an ice bath under a nitrogen or argon gas environment.

■ **PAUSE POINT:** If DCC is used as a coupling agent, a white precipitate will appear due to the formation of an insoluble acyl urea byproduct leading to white precipitate, EDC does not form such a precipitate because its urea byproduct is generally soluble in dichloromethane.

17. Check if Steglich esterification of chemotherapeutic into prodrug took place by completing thin layer chromatography (TLC) on chemotherapeutic-azide to the reaction mixture as follows:

- i. Deposit 5 drops of each sample 1 cm from the bottom of the TLC plate using capillary glass tubing adding one drop at a time, allowing each drop to dry before adding an additional drop.
- ii. Place 1 ml of TLC solvent (50% n-Hexane/50% Ethyl acetate [vol/vol]) into the bottom of a chromatography glass chamber and cover it with the lid to minimize the evaporation of the volatile solvent.
- iii. Insert the TLC plate into the chromatography glass chamber and cover it with the lid, making sure sample spots are not submerged in TLC solvent.
- iv. Allow solvent to run up the TLC plate until reaching ~5 mm from the top of the TLC plate.
- v. Remove the TLC plate from the chamber allowing it to air dry and image UV shadow to check compound migrations using a UV lamp.

!**CAUTION:** TLC should be completed in a vented hood to prevent exposure to hazardous chemicals.

▲**CRITICAL STEP** Additional staining with 5% sulfuric acid in 95% ethanol (vol/vol) may be needed to confirm the synthesis of the prodrug and detect any impurities that may appear during the reaction to ensure the purity of the prodrug. To perform the staining, spray the TLC plate with 5% sulfuric acid in 95% ethanol (vol/vol) and dry it in the oven for 5–10 minutes.

▲CRITICAL STEP The R_f value of both substrate drug and prodrug can be calculated during the TLC plate experiment for quality control purposes. The R_f value is calculated as $R_f = \text{distance traveled by solute} / \text{distance traveled by solvent}$. Distance should be measured and quantified between defined start and end lines (1 cm from the bottom of the plate and 5 mm from the top of the plate, respectively). When the solvent reaches the end line, pull the plate out and measure the R_f of both substrate drug and prodrug. An R_f number for either substrate drug or prodrug between 0.7 and 0.3 is considered acceptable. The R_f value difference between the substrate and prodrug is expected to be larger than 0.2, with the prodrug (chemotherapeutic-azide) having a larger R_f value over chemotherapeutics and all reactants.

18. Upon confirmation of reaction completion, concentrate the reaction by evaporating on a rotary evaporator. Then, redissolve dried prodrug in 10 ml ethyl acetate, to form a white precipitant of DCU. Filter DCU precipitant with filter paper and wash the organic fraction with saturated Sodium Bicarbonate solution and Brine.

▲CRITICAL STEP: Filtering is not necessary if using EDC as a coupling agent.

19. Dry the organic fraction over sodium sulfate anhydrous to absorb the water or moisture. Filter through filter paper and concentrate by rotary evaporator.
20. The purification of prodrug from chemotherapeutic and azide linker is critical in order to ensure click chemistry reaction with RNA and full removal of DCU. Purify chemotherapeutic prodrugs from reactants and byproducts via CombiFlash (Teledyne LABS) (Option A) or silica (SiO_2) column chromatography (Option B) as follows:
 - A. CombiFlash (Teledyne LABS).
 - i. Follow equipment set up in Box 3.
 - ii. Load 100 mg of chemical into the CombiFlash silica column and air dry the column.
 - iii. Use N-Hexane (solvent A) and Ethyl acetate (solvent B) at different specific ratios to wash off the product from the CombiFlash silica column starting the wash with 100% solvent A for 3 minutes.
 - iv. Gradually change the ratio of solvents from 100% solvent A/0% solvent B (vol/vol) to 13% solvent A/87% solvent B (vol/vol) over 10 minutes. Prodrug will elute off the column through the solvent gradient (i.e. SN38- N_3 washes off at 13% solvent A/87% solvent B during the 5-minute plateau wash).
 - v. Purified prodrug is automatically collected and re-concentrated by removing the solvent. Also collect remaining samples that are eluted out at 80% DCM/20% Methanol (vol/vol) during the cleanup and shutdown procedures of the CombiFlash in Box 3 to ensure samples are in the collect product and not discarded here.
 - B. Silica (SiO_2) column chromatography.
 - i. In a glass column with a stopcock on the lower end fitted with glass wool, close the stopcock and fill the column with dry silica gel.
 - ii. Slowly fill 3/4 of the column with solvent (90% n-Hexane:10% Ethyl acetate [vol/vol]). Open the stopcock and let the solvent pass through the silica gel. Pack the silica gel by applying pressure gas from the top of the column while the solvent passes through. Repeat the process several times until the silica gel is

- packed, drain excess solvent until just above the silica stationary phase, and close the stopcock.
- iii. Add silica gel to a round-bottom flask containing a concentrated solution of the mixture of prodrug until a slurry is formed (add 2 – 4 mL Ethyl Acetate if necessary). Concentrate using a rotary evaporator until dry.
 - iv. Add the dry silica gel containing the prodrug to the column containing packed silica gel and cover it with glass wool.
 - v. Elute samples using the same solvent system used in TLC (90% n-Hexane:10% Ethyl acetate [vol/vol] to 50% n-Hexane:50% Ethyl acetate [vol/vol]), thus byproducts elute off the column followed by product. Collect and fractionate elution and test for which fraction has prodrug product via TLC as in step 17.
21. Combine all fractions with the product and concentrate via rotary evaporator.
22. Chemotherapeutic prodrug is now considered pure and ready for conjugation to RNA oligo.
- **PAUSE POINT:** Chemical prodrug can be stored at -20°C until further use for 1 year.
23. Verify chemotherapeutic prodrug formation by NMR and mass spectrometry (**Fig. 7**) demonstrating the presence of ester formation and the presence of 1-azidohexane linker (Option A) or corresponding mass increase (Option B), respectively.
- A. NMR of chemotherapeutic prodrug (**Fig. 7A**).
- i. Dissolve 2 – 4 mg of the prodrug in 0.5 mL into an appropriate deuterated solvent (i.e. deuterated chloroform, CDCl₃)
 - ii. Transfer the prodrug solution into the dry NMR tube and cap the NMR tube to prevent evaporation.
 - iii. Launch the Bruker Topspin 2.1 program. Type **edc** into the command line on bottom left of the software interface to create a new experiment and choose the appropriate NMR experiment (i.e. proton, carbon, etc.) in the instrument.
 - iv. Place the NMR tube into the spinner and adjust it to the correct sample height. Use the depth gauge in the spinner to set the correct depth
 - v. Type **ej** into the command line to create airflow in the sample loader. Place the sample in the NMR instrument and type **ij** (inject sample) to lower the NMR tube containing the sample into the magnetic field
 - vi. Type **lock** to lock the solvent signal of the sample.

▲ **CRITICAL STEP:** The solvent signal must match the solvent used to dissolve the prodrug in step 23(A)i. Failure to lock the correct solvent or using the wrong solvent will result in a shift of the signal to an incorrect ppm.
 - vii. Perform necessary tuning by typing **atma** into the command line, followed by shimming by typing **topshim** into the command line to optimize the magnetic field and produce a good spectrum.
 - viii. Retrieve the solvent information by typing **getprosol** into the command line.
 - ix. Set the number of the scans by typing **ns** into the command line and adjust the scan as necessary.
 - x. Begin data acquisition by typing **rga** and **zg** into the command line.
 - xi. Once the experiment is finished type **efp** and **apk** into the command line to process NMR data.
 - xii. Type **ej** into the command line to retrieve back the sample.

- xiii. Gently lift the NMR tube and spinner from the loader.
 - xiv. Stop the airflow from the loader by typing **ej** into the command line.
 - xv. Further analyze NMR data in Mestrelab Nova or other NMR software following manufacturer's guidelines.
- B. Mass Spectrometry of chemotherapeutic prodrug (**Fig. 7B-C**).
- i. Prepare the prodrug sample for mass spectrometry by dissolving 100 µg of prodrug in 1 ml of 50:50 (vol:vol) mixture of methanol and H₂O. Place the sample into an HPLC glass vial. Also, prepare control samples (blank sample consisting of solvent only and native chemotherapeutic drug dissolved in the same solvent).
 - ii. Launch MassLynx software using, then, select **shortcut**, **instrument**, and then **inlet method**.
 - iii. Launch OA Manager from the MassLynx window by choosing **shortcut**, then **openlynx**, and then **OA Manager**
 - iv. Launch OA Login from the shortcut on the desktop.
 - v. Prime the pump by selecting **ACQUITY additional status** then right click in the section "**Binary Solvent Manager**".
 - vi. Select Prime A/B solvent, then wait for 2 minutes.
 - vii. Submit the samples by opening OALogin window and clicking sample. Follow the instructions provided by the wizard.
 - viii. Pick methods and load samples to the Waters Acquity UPLC and ESI-MS following manufacturer's guidelines. Run the samples.

[H2] Conjugation of prodrug-azide to 4WJ component strand via click chemistry
TIMING: 4-5 d.

- 23. Dissolve prodrug in click solvent to ensure solubilization of poorly aqueous soluble drugs. Ensure reaction does not become turbid with a minimum ratio of 50% water and 50% organic solvent (vol/vol).
 - ▲ **CRITICAL STEP** If reaction becomes turbid or a precipitant is seen add more click solvent to reaction.
- 24. For incorporation of prodrug-azide into alkyne-modified RNA, a ratio of 5 prodrugs to 1 alkyne modification is generally desired. That means a ratio of 30 prodrugs to 1 RNA is required. Add desired amount of RNA to dissolved prodrug for each 4WJ component strand reaction (4WJ_A, 4WJ_B, 4WJ_C, 4WJ_D).
 - ▲ **CRITICAL STEP** The ratio of prodrug to RNA/alkyne modification can be varied and titrated across multiple ratios to test the best conjugation methodology.
- 25. Add click solution (composed of CuBr, TBTA, and ascorbic acid) to the reaction at a ratio of 1:20 of alkyne drug to CuBR and vortex reaction for 30 sec.
 - ▲ **CRITICAL STEP:** Copper II (Cu²⁺) is required to catalyze this reaction but increases reactive oxygen species and free radicals that can be quite damaging to biological samples, including RNA. Cu²⁺ ions can promote the backbone cleavage of RNA strands or permanent base modification.^{206,207} It is critical that the time of incubation of Cu²⁺ with RNA is controlled, free Cu²⁺ is removed following the reaction (see step 30) and the quality of the RNA strands is confirmed following click reactions by gel electrophoresis as described in Step 31.
- 26. Incubate reaction at room temperature overnight.

▲ **CRITICAL STEP** If reaction is not completed or turbidity is seen, reaction can be heated to 37°C. Heating reaction over a prolonged time period may promote RNA degradation.

■ **PAUSE POINT:** Click reaction of chemical drug and RNA oligo can be paused and stored at -20°C for up to 6 months.

27. Ethanol precipitate RNA products from click chemistry reaction to remove click solvent and excess copper catalyst by adding 2.5 volumes of 100% ethanol and 0.1 volume 3M sodium acetate. Allow precipitate to occur overnight at -20°C.

28. Centrifuge the mixture at 16,500 g for 30 min at 4°C and remove the supernatant; wash the pellet with 70% (vol/vol) ethanol and dry the pellet for 5 min with a SpeedVac.

29. Rehydrate the pellet in DEPC treated H₂O.

■ **PAUSE POINT:** Click reaction of chemical drug and RNA oligo can be paused and stored at -20°C for up to 6 months.

30. Purify RNA oligos conjugated with prodrug in order to remove non-reacted prodrug, unlabeled oligos, and copper catalyst, following either option A or B.

a. Purify the 4WJ component strands from Step 29 by 15% (wt/vol) 8 M Urea denaturing PAGE gel run in TBE buffer.

i. Add RNA to an equal volume of 2× denaturing loading dye and load samples into the wells of a 15% (wt/vol) 8 M Urea denaturing PAGE gel. Run the gel at 120 V for ~100 min.

ii. Excise the band corresponding to conjugated 4WJ component strand under UV light (254 nm), cutting the band into small (~2 × 2 mm) pieces. Elute the RNA using RNA elution buffer by completely submerging gel pieces in buffer in a microcentrifuge tube at 37°C for 4 hours.

iii. Pipette the supernatant from the elution step while leaving gel pieces and add it to 2.5 volumes of 100% ethanol and add 10% volume 3M sodium acetate. Allow precipitate to occur overnight at -20°C

iv. Centrifuge the mixture at 16,500 g for 30 min at 4°C and remove the supernatant; wash the pellet with 70% (vol/vol) ethanol and dry the pellet for 5 min with a SpeedVac.

v. Rehydrate the pellet in DEPC treated H₂O of desired volume.

b. Purify the 4WJ component strands from Step 29 by reverse-phase HPLC (**Fig. 8** left panel). Follow the HPLC equipment setup procedure (Box 2) to set up the system and prepare for RNA loading and purification.

i. Following the conditioning the column, set the signal baseline to zero in the “Online Plot” tab of the software by clicking the “Balance” button.

ii. Load an RNA purifying methods in the HPLC software by clicking “Method” tab on the top bar and select the desired method for RNA purification. The parameters for the methodology should be as follows: The first 10 minutes will be a constant wash with a mixture of 95% of A and 5% of B (vol/vol) at 1 ml/min flow rate. A continuous change of mixture from 95% A and 5% B to 5% A and 95% B (vol/vol) will be set during the next 60 minutes at 1 ml/min. The final 10 minutes will be a constant wash with a mixture of 5% A and 95% B (vol/vol) at 1 ml/min.

- iii. Input the sample information into the HPLC software by clicking “RunControl” and select “Sample Info” to input sample information and specify the saving folder.
- iv. To initiate the loading of RNA sample into the injection loop, set the injector to LOAD and load 500 μ L of 100% acetonitrile into the injector three times. Continue washing the loop with 500 μ L of HPLC-grade H₂O three times to flush out the acetonitrile.
 - !Caution:** Always flush with water after acetonitrile to prevent acetonitrile residue from interfering with sample interaction with the column.
- v. Click the “Method” tab on the software and select “Save Method” before running. Ensure any changes to the method are saved before proceeding.
- vi. Set up the fraction collector by resetting the fraction collector to its initial position. Right-click on the fraction collector module and select “Reset Fraction Collector”. Turn on collection by selecting the method and changing the setting from “off” to “Time-based with timeslices” (0.5 min or 1 min) or “Peak-based” collection. Choose “Assign Wellplates” to assign the blocks for 96-well plates. Set up clean 96-well collection plates in the fraction collector blocks.
- vii. Inject and load the RNA sample into the injector using a clean glass syringe. Flip the injector valve to the INJECTION position to automatically start the run. The drug-labeled RNA will be separated from unreacted RNA-6 alkynes using an 95% H₂O TEAA/5% acetonitrile TEAA to 5% H₂O TEAA/95% acetonitrile TEAA (vol/vol) gradient controlled by the method selected in the software. The unreacted RNA-6 alkynes will be collected at around 70% H₂O TEAA/30% acetonitrile TEAA (vol/vol), and the conjugated RNA-6 drugs will be collected at around 20% H₂O TEAA/80% acetonitrile TEAA (vol/vol). Samples will be automatically collected, and fractions are collected based on the wash-off time.
- viii. At the completion of the run move the injector back to the LOAD position. Collect fractions from the fraction collector for analysis. Shut down the HPLC following the manufacturer’s recommended procedure and shutdown procedure in the Equipment Setup section.
- ix. Analyze the 260 nm absorbance of fractions under the “Data Analysis” tab of the software. Select the peaks of interest and keep the corresponding fraction numbers for further processing.
 - !Caution:** If there are multiple peaks of purified RNA on the 260 nm absorbance and uncertainty in which peak corresponds to the drug conjugated 4WJ strand, save all peaks and perform further gel analysis (as in Step 38 above without excising bands) to determine the actual peak of interest. Image gel stained with ethidium bromide on a gel imager such as Typhoon FLA 7000 (GE Healthcare).
 - ▲ CRITICAL STEP:** Ethidium bromide is toxic and alternative fluorescent nucleic acids dye can be used a safe alternative. SYBR Green II dye is specifically designed for RNA intercalation and can be used to stain gels.
- x. Dry the collected fractions using a SpeedVac overnight. Once samples are dried, resuspend the fractions containing RNA products with DEPC-treated H₂O. Concentrate the RNA by loading 500 μ L of the RNA product into an Amicon® Ultra Centrifugal Filter with a 10 kDa MWCO and a 0.5 mL sample volume. Spin at

14,000 g for 15 minutes and collect the fraction from the filter device sample reservoir. RNA is now purified and ready for future steps.

31. Confirm chemical drug conjugation to 4WJ oligo strand by running a 15% (wt/vol) Urea denaturing PAGE gel in TBE buffer (**Fig. 8** middle panel).

- i. Mix ~250 ng of RNA conjugated with chemical drug (including RNA with no drug controls) with an equal volume of 2× running buffer to generate samples to be loaded into the gel.
- ii. Prepare and run 15% (wt/vol) Urea denaturing PAGE in TBE buffer of RNA samples at 150 V for 1 h.
- iii. Stain gel in ethidium bromide (or alternative as above) containing TBE buffer and visualize on gel imager, ie. Typhoon FLA 7000 (GE Healthcare).

■ **PAUSE POINT:** RNA oligos can be stored at -20°C for up to 6 months or at -80°C for long term (>1 year) storage.

[H2] Assembly of ultra-stable 4WJ RNA nanoparticles harboring chemotherapeutics TIMING: 2-3 d.

32. The 4WJ RNA nanoparticle assembles into an ultra-stable complex consisting of the four component strands. Combine 4WJ_A, 4WJ_B, 4WJ_C, and 4WJ_D strands at an equimolar ratio in TES buffer. Then, denature RNA strands at 95°C for 5 min followed by gradual cooling to 4°C over the course of 1 hour in a thermal cycler.

33. Assay the assembly of the 4WJ nanoparticle using a 12% (wt/vol) native PAGE gel run in TBE buffer at 120 V for 1 hour and compare its migration to single strands and partial 4WJ assemblies. Stain gel in ethidium bromide (or alternative as above) containing TBE buffer and visualize on gel imager, ie. Typhoon FLA 7000 (GE Healthcare) (**Fig. 9A**).

34. Purify the 4WJ assemblies by 12% (wt/vol) native PAGE gel run in TBE buffer at 150 V for 1 h. Excise the band corresponding to assembled 4WJ construct under UV light (254 nm). Elute the RNA using RNA elution buffer containing 10 mM Mg²⁺ at 37°C for 4 hour as in Step 30(a)ii.

35. Take the supernatant from the elution step and add it to 2.5 volumes of 100% ethanol and add 0.1 volume 3M sodium acetate. Allow precipitate to occur overnight at -20°C

36. Centrifuge the mixture at 16,500 g for 30 min at 4°C and remove the supernatant; wash the pellet with 70% (vol/vol) ethanol and dry the pellet for 5 min with a SpeedVac.

37. Rehydrate the pellet in TES buffer of desired volume (normally aiming for ~1 µg/µl).

■ **PAUSE POINT:** RNA nanoparticles can be stored at -20°C for up to 6 months or at -80°C for long term (>1 year) storage. RNA nanoparticles formation and stability should be assayed as in steps 38 - 40 below prior to use after long term storage.

[H2] Characterize 4WJ-drug conjugate nanoparticles and assay drug conjugation. TIMING: ~1 month.

▲ **CRITICAL:** The following assays provide a rather complete characterization of the 4WJ RNA nanoparticles; however, the user may decide all studies are not necessary for their use. Confirming 4WJ nanoparticle assembly by native PAGE should be done by all users to at least ensure nanoparticle formation and drug conjugation.

[H3] Confirm 4WJ nanoparticle assembly by 12% (wt/vol) native PAGE in TBE buffer

38. Mix ~250 ng of RNA nanoparticles from Step 37 (including controls such as monomer strands, dimer, and trimer intermediates, and 4WJ without drug as in **Fig. 9A**) with 6× running buffer to 1× to generate samples to be loaded into the gel.
39. Prepare and run 12% (wt/vol) native PAGE in TBE buffer of RNA samples at 150 V for 1 h.
40. Stain gel in ethidium bromide (or alternative as above) containing TBE buffer and visualize on gel imager, ie. Typhoon FLA 7000 (GE Healthcare). See Fig 3b and 9a.

[H3] Nanoparticle size measurement by dynamic light scattering (DLS)

41. Dissolve RNA nanoparticles in TES buffer to a final concentration of 1 μM. Measure and analyze the hydrodynamic radius of RNA nanoparticles using Malvern Zetasizer Nano-SZ at 25°C and laser wavelength 633 nm (according to the manufacturer's guideline). See Fig. 3d and 9b,c.

[H3] Determination of enzymatic stability and drug release profile

42. Incubate 1 μM preassembled RNA nanoparticles from Step 37 in Dulbecco's Modified Eagle Medium (DMEM) cell culture media containing 50% (vol/vol) fetal bovine serum (FBS) [for enzymatic stability] or DEPC treated H₂O at 37°C for 0, 1, 2, 4, 8, 12, 18, 24, 36, and 48 h timepoints, flash freezing each timepoint on dry ice to halt reactions.
43. Mix each timepoint with 6× running buffer and load samples into a 3% (wt/vol) agarose gel in TAE buffer containing ethidium bromide (or alternative as above). Run the gel at 100 V for 40 min.
44. Visualize on gel imager and quantify the intensity of each 4WJ band in relation to the 0 h timepoint intensity using ImageJ and plot 4WJ assembly percentage against time. The nuclease degradation of RNA nanoparticle will demonstrate a complete decrease in RNA visualized at each timepoint, while drug release in water from the RNA nanoparticle will demonstrate a more rapid migration rate in the gel.

[H3] Determination of nanoparticle annealing temperature (T_a) by RT-PCR

45. Conduct melting curve experiments by monitoring the fluorescence of the RNA nanoparticles using the Roche LightCycler 480 real-time PCR system. Add preassembled RNA nanoparticles to a white, round bottom 96-well plate in TES buffer at a final concentration of 2.5 μM and 1 μM in 1× SYBR Green II nucleic acid dye (emission 465–510 nm), which binds double-stranded but not single-stranded nucleic acids.
46. On the Roche LightCycler 480 RT-PCR, heat samples to 95 °C for 5 min to denaturing process followed by a slow cooling ramp to 20 °C at a rate of 0.11 °C/s as an annealing process, while monitoring SYBR Green II emission. Analyze data with the LightCycler 480 analysis software (according to the manufacturer's instructions) by using the first derivative of the melting profile.
47. Represent annealing curves to show the annealing temperature T_a using the first derivative of the melting profile (follow manufacturer's guidelines). Represent the melting temperature (T_m) value using the mean and standard deviation from at least three independent experiments.

[H3] Determination of nanoparticle T_m by temperature gradient gel electrophoresis (TGGE)

▲ **CRITICAL:** TGGE allows for the determination of the melting profile and temperature (T_m) of a single nanoparticle (**Fig. 9G** left panel).

48. Mix ~250 ng of RNA sample with 6× running buffer to 1× to generate one sample per well and load the same RNA nanoparticle across each of the 10 wells for the temperature gradient.
49. Run in a 12% (wt/vol) native PAGE in TBE buffer at 100 V for 10 min at room temperature to allow RNA samples to penetrate into the gel.
50. Transfer the gel to the Biometra TGGE according to manufacturer's instructions with the temperature gradient applied perpendicular to the electrophoresis current. Temperature gradient can be varied but selecting 30-80°C provides a wide range to allow calculating the T_m . Run the gel at 20 W for 60 min at room temperature.
▲CRITICAL STEP: Elevated temperatures of TGGE can dry the PAGE gel out during electrophoresis. Ensure electrophoresis wicks are properly covering the gel ends and keeping the gel hydrated.
51. Stain gel in ethidium bromide (or alternative as above) containing TBE buffer and visualize on gel imager, ie. Typhoon FLA 7000 (GE Healthcare). Quantify the intensity of each 4WJ band in relation to total lane intensity using ImageJ and plot 4WJ assembly percentage against temperature. The T_m value is defined as the temperature at which 50% of nanoparticle is dissociated.

[H2] Characterize therapeutic effects of 4WJ-drug conjugate nanoparticles *in vitro* (TIMING: ~ 1 month.

▲CRITICAL: RNA nanoparticles functionalized with an RNA aptamer allow specific binding and uptake by cancer cells overexpressing the correlating receptor. This binding can be monitored through the use of a fluorescent label (ie. Alexa fluor 647) on a component strand of the 4WJ nanoparticle and imaging this fluorophore following incubation with respective cancer cells. Additionally, following binding and cellular internalization, the RNA nanoparticles are able to deliver conjugated chemotherapeutics. The below procedures describe different assays to monitor the targeting of TNBC cells (MDA-MB-231) by an EGFR aptamer on the 4WJ to deliver conjugated paclitaxel.³⁹

[H3] *In vitro* assay of RNA nanoparticle binding to tumor cells via flow cytometry

52. Maintain MDA-MB-231 cells in DMEM/F12 culture medium with 10% FBS and 1% penicillin/streptomycin. Trypsinize cells and rinse them with in DMEM/F12 without FBS once. Label 4WJ RNA nanoparticles with Alexa647 fluorophore as previously described with 5'-NH₂ modifications and Alexa647-NHS Ester.^{39,67} Incubate 2×10^5 cells with Alexa647-labeled 4WJ RNA nanoparticles harboring EGFR aptamer or with control RNA nanoparticles not harboring the aptamer, as well as a cell only control at 37°C for 1–4h in centrifuge tubes. RNA nanoparticles are typically used at 100 nM, but this concentration can be varied.
53. Pellet cells following 4WJ incubation by centrifuging at 1000 g for 5 m at room temperature and wash cells with 500 µl PBS.
54. Repeat pelleting and resuspend cells in 250 µl PBS buffer and assay them by flow cytometry using a Biosciences LSRII flow cytometer for Alexa647 (Emission: 650 nm, Excitation: 665 nm) following manufacturer's guidelines.

[H3] *In vitro* assay of RNA nanoparticle binding to tumor cells via confocal microscopy

55. Maintain MDA-MB-231 cells as described in Step 52. Trypsinize cells and seed cells to 50% confluency on glass cover slips in complete culture medium overnight.

56. Incubate cells with 100 nM final concentration of Alexa647-labeled 4WJ RNA nanoparticles harboring EGFR aptamer or with control RNA nanoparticles not harboring the aptamer, as well as a cell only control at 37°C for 4 h.
57. Wash cells twice with PBS and fix cells with 4% (wt/vol) paraformaldehyde at room temperature for 30 min.
58. Wash cells with PBS and treat with 0.1% Triton X-100 in PBS buffer for 5 min.
59. Wash cells with PBS and stain cells using Alexa Fluor® 488 phalloidin (follow manufacturer's guidelines) to label the cytoskeleton.
60. Mount the cells using ProLong® Gold Antifade Reagent to stain the nucleus.
61. Assay for aptamer binding and cell entry with an Olympus FV3000 confocal microscope following manufacturer's guidelines imaging each of the three fluorophores. Generate overlay images using manufacturer's software to determine nanoparticle binding and internalization.

[H3] *In vitro* assay of RNA nanoparticle induced inhibition of tumor cell proliferation via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

62. Maintain MDA-MB-231 cells as described in Step 52 above. Trypsinize cells and seed 5×10^3 cells per well in a 96-well plate overnight in complete culture medium.
63. Incubate 4WJ RNA nanoparticles with and without paclitaxel (PTX) and/or EGFR aptamer and free PTX into each well in triplicates at indicated concentrations (0.25 – 24 μ M) for 48 h at 37°C in a humidified 5% CO₂ incubator.
64. Using the Promega CellTiter 96 Non-Radioactive Cell Proliferation Assay follow manufacturer's guidelines adding 15 μ l of MTT Dye Solution to each well and incubate at 37°C in the dark for 4 h.
65. Add 50 μ l of Solubilization Solution/Stop Mix to stop the dye solution reaction and solubilize the formed crystals until solution is uniformly colored.
66. Measure the absorbance of each well at 570 nm using a plate reader (Bio-Tek Synergy 4), averaging the triplicate samples. Normalize absorbance to cell only sample to estimate cell proliferation inhibition causes by PTX release from the RNA nanoparticles.

[H3] *In vitro* assay to measure RNA nanoparticle induced apoptosis of tumor cells via Annexin V/PI staining

67. Maintain MDA-MB-231 cells as described in Step 52 above. Trypsinize cells and seed 5×10^4 cells per well in a 24-well plate overnight at 37°C and culture in a 5% CO₂ humidified incubator.
68. Incubate cells with 4WJ RNA nanoparticles with and without PTX and/or EGFR aptamer and free PTX at a final concentration of 1 μ M of PTX for 24 h at 37°C in a humidified 5% CO₂ incubator.
69. Trypsinize cells into suspension, wash in PBS buffer twice and suspend in 100 μ l 1 $\times$ Annexin V-FITC binding buffer using the BD Pharmingen FITC Annexin V Apoptosis Detection Kit. Add 5 μ l of Annexin V-FITC followed by 5 μ L of propidium iodide (PI) to each well and incubate at room temperature for 20 min.
70. Transfer samples to tubes for flow cytometry with 200 μ L of 1 $\times$ binding buffer and assay samples by flow cytometry using a Biosciences LSRII flow cytometer following manufacturer's guidelines.

[H3] *In vitro* assay of RNA nanoparticle induced cytokine response

71. RAW 264.7 macrophage cells are commonly used to examine cytokine induction and release. Maintain RAW 264.7 cells in DMEM full growth medium with 10% FBS. Aliquot 5×10^4 cells per well into a 24-well plate and cultured at 37°C overnight in a humidified 5% CO₂ incubator.
72. Incubate 4WJ RNA nanoparticles and lipopolysaccharide (LPS, 5.5 µg/mL, equal amount as 100 nM 4WJ RNA nanoparticles) per well of RAW 264.7 cells in 200 µl Opti-MEM cell medium at 37°C for 16 h.
73. Collect the supernatants of cell medium and freeze at -80°C for further analysis. The TNF-α and IL-6 in diluted supernatants were measured by Mouse ELISA MAX Deluxe sets following manufacturer's protocols.

[H1] TIMING

Steps 1 – 13, Synthesis of 4WJ component strand oligos: ~1 wk.

Steps 14 – 22, Chemical synthesis of chemotherapeutic prodrug: ~1 wk.

Steps 23 – 31, Conjugation of prodrug-azide to 4WJ component strand via click chemistry: 4-5 d.

Steps 32 – 37, Assembly of ultra-stable 4WJ RNA nanoparticles harboring chemotherapeutics: 2-3 d.

Steps 38 – 51, Characterize 4WJ-drug conjugate nanoparticles and assay drug conjugation: ~1 m.

Steps 52 – 73, Characterize 4WJ-drug conjugate nanoparticles for therapeutic effect *in vitro*: ~1 month.

[H1] TROUBLESHOOTING

Step	Problem	Possible Problem	Possible Solution
13	Low yield in RNA oligo synthesis.	- Water contamination in phosphoramidites or solvents. - Inert gas supply (argon or helium) has too high humidity. - Low gas (argon or helium) pressure.	- Use new solvents that have been dehydrated through use of molecular sieves, degassed, and dissolve all phosphoramidites in dry environment (i.e. under argon gas). - Check with gas supplier on humidity levels of gases and purchase low humidity inert gas or include an inline desiccant column. - Check system for gas leaks and complete preventative maintenance to Biosset ASM-800 according to manufacturers recommendations.
18	Low yield in prodrug formation.	- Reaction time is too short. - Water contamination in reaction. - Reaction needs additional catalysis.	- Extend time of reaction. - Ensure reaction is under dry environment (i.e. in argon gas) or use molecular sieves to absorb water. - Replace DCC with EDC in the reaction.
23	NMR signal of azide conjugated prodrug is obscured by impurities.	- Presence of unreacted linker or prodrug resulting in overlap with prodrug signals. - Presence of trace amounts of Ethyl acetate, Dichloromethane or Methanol solvents in samples. - Presence of moisture or residual water can result in a broad peak from 3.0 – 3.5 ppm in CDCl₃.	- Complete additional column chromatography to remove reactants. - Thoroughly dry samples either under vacuum for 12 hour or using a Schlenk line for several hours prior to running NMR.

29	Poor recovery of RNA following ethanol precipitation.	• RNA not fully eluted from gel pieces. • Incorrect final concentration of ethanol preventing RNA precipitation. • Too low concentration of RNA.	• Extend elution time at 37°C or repeat elution and cut gel into smaller pieces to encourage buffer exchange. • Ensure solution has at least 70% (vol/vol) ethanol. RNA will not precipitate at lower ethanol concentrations. • Reduce total volumes to increase RNA concentrations, thus promoting aggregation.
31	Low reaction yield in prodrug conjugation to RNA oligo.	• Low concentration of Cu¹⁺ ions in reaction. • Reaction is turbid and prodrug is not dissolved.	• Ensure CuBr solution is freshly prepared. • Ensure proper CuBr to TBTA ratio or increase ratio of TBTA to CuBr to ensure Cu²⁺ is converted to Cu¹⁺. • Increase concentration of Click solvent, heat reaction to 37°C or sonicate reaction. Note: prolonged exposure to heat may promote RNA degradation.
40	Poor 4WJ nanoparticle formation	• Incorrect Mg²⁺ concentration. • Incorrect molar ratios of RNA oligos	• Excessive Mg²⁺ concentration can promote nanoparticle aggregation. Change buffer conditions to reduce Mg²⁺ concentrations. • If byproducts (trimer, dimer, or monomer) bands are seen in the gel, equal molar mixing of RNA oligos was not achieved and concentrations of oligo strands should be checked.
51	Uneven migration rates during TGGE electrophoresis	• Drying of the gel and buffer wicks due to gel heating gradient.	• Ensure wicks are saturated with buffer, submerged in buffer chambers, and have proper overlap with gel to prevent drying.
54, 61	Poor cancer cell binding	• Concentration of RNA nanoparticle used too low. • Incubation time of RNA nanoparticles with cells is too long or short.	• Increase concentration of RNA nanoparticle incubated with cell (i.e. 250 nM). • Increase incubation time of RNA nanoparticles with cells if too little binding is seen. • Decrease incubation time of RNA nanoparticles with cells if no ligand control shows too high of binding with cells.

[H1] ANTICIPATED RESULTS

This protocol provides detailed procedures for the design and construction of multifunctional, stable RNA nanoparticles capable of selectively targeting solid tumors and conjugating up to 24 molecules of chemotherapeutic drugs, including paclitaxel, SN38, and camptothecin. We also provide methodology for characterizing the RNA nanoparticles and assaying for *in vitro* cancer cell proliferation inhibition and toxicity. The inclusion of a tumor targeting ligand (e.g. EGFR aptamer), inclusion of a fluorophore (e.g. Alexa fluor 647), and conjugation of the chemotherapeutic drug occurs prior to final assembly of the 4WJ RNA nanoparticle through RNA synthesis and click chemistry reactions. The chemical reactions for chemotherapeutic modification and conjugation to the RNA were selected for their relative high yield and easy purification from byproducts. The anticipated results of this protocol are as follows:

1. 4WJ RNA nanoparticles are highly thermodynamically (**Fig. 3e**) and chemically stable (**Fig. 9d**) and form into homogeneous nanocomplexes (expected to have a hydrodynamic size of ~10 nm) (**Fig. 3d** and **Fig. 9c**). The 4WJ nanoparticle is designed to have a high

thermodynamic stability through strong base pairing of component strands that are not interrupted by the conjugation of hydrophobic chemical drugs (**Fig. 3e** right panel).³⁹ Additionally, the pRNA-3WJ, which served as the basis for designing the 4WJ, has shown strong stability against chemical denaturation, including 8 M urea.⁵⁴ Upon the inclusion of 2'-Fluoro modifications to pyrimidine nucleotides during chemical synthesis, the RNA nanoparticle is resistant to nuclease cleavage and degradation.^{39,54,185,208} The base pairing of the 4WJ allows for specific interactions of component strands that result in controlled stoichiometry generating a homogeneous product (**Fig. 3b-c**).

2. It is anticipated the synthesis of RNA component strands using a 1 μ mol scale Universal Support Column should produce ~4 mg of each RNA strand. If concentrations of RNA strands are substantially less, the most common cause is either a pressure leak within the synthesizer causing volumes to be inaccurately pumped across the support column or water contaminants in the phosphoramidite or solutions used during the synthesis. Users should check that all bottles are tightly fit onto the synthesizer and pressure maintained during the entire synthesis. If water is the cause of low yield, solvents can be dried and molecular sieves used to absorb any water present. To monitor yield of each nucleotide addition on the support column, users should check for a vibrant orange color across the column indicating deprotection of each nucleotide (Step 3iv) and release of DMT.

During purification of chemotherapeutic conjugated RNA component strands by HPLC, a high yield (>90%) of total RNA should be recovered; while gel purification results in a lower (~75%) final yield due to inefficiencies in gel elution. If low yields are seen in HPLC purification, it is most likely due to poor RNA adhesion to the column due to improper column conditioning. Users should recheck the equipment setup protocol. If a large amount of RNA is lost by gel purification issues could be due to poor gel elution yield or poor ethanol precipitation conditions. Users must make sure RNA samples are precipitated using 70% ethanol for maximum efficiency. Gel elution can be improved through repeated elutions, longer elution time, or cutting the gel piece into smaller pieces to promote buffer exchange.

3. Due to the reactive hydroxyl groups on the suggested chemotherapeutics here, azide linkers can be incorporated onto the small molecule drugs generating prodrugs that are conjugatable to RNA nanoparticles. Successful modification of these chemotherapeutics can be confirmed by ¹H-NMR and mass spectrometry. Conjugation of the azide linker (6-Azido Hexanoic Acid) forming into the prodrug is confirmed by the appearance of the new peak in the aliphatic region (2 – 5 ppm) of the ¹H-NMR (**Fig. 7A**). The 6-Azido Hexanoic acid moiety shows two distinct triplets (with integration of 2H each) corresponding to methylene proton adjacent to N₃ and ester group. The ester group appears slightly downfield (shift to higher ppm) due to deshielding by the electronegative ester group compared to another triplet that is adjacent to the Azide. A quintet (2H) is observed for the central methylene protons, and a multiplet (4H) corresponds to the two internal methylene groups between the terminal triplets and quintet. The ¹H-NMR spectrum can also be used for assessing the purity of the azide conjugated prodrug. Successful conjugation is also confirmed by the disappearance of the carboxylic acid signal from 6-azidohexanoic acid, indicating ester bond formation. The free carboxylic acid typically appears as a small, broad peak around 10–12 ppm in the ¹H NMR spectrum. Upon conjugation, this peak disappears due to esterification. The carboxylic acid proton is exchangeable with water or

moisture in deuterated solvents, which can cause the peak to broaden or become undetectable).

4. The 4WJ RNA nanoparticle with inclusion of 2'-Alkyne modifications results in specific conjugation of chemotherapeutic prodrugs (**Fig. 8**).^{34,39-41,61} The resulting conjugates do not allow for aggregation of chemical drugs nor interference with nanoparticle complexation. The 4WJ has been designed to space chemical drug conjugates to prevent these two issues, while having a high yield of conjugation. Additionally, the conjugation of hydrophobic drugs to the RNA 4WJ results in substantial water solubility of the drugs (**Fig. 8** right panel).
5. The 4WJ RNA nanoparticle is designed so that base pairing of component strands only allows for formation into >10 nm nanoparticles (**Fig. 3d** and **Fig. 9c**) and generally nanoparticles do not form aggregates or larger particles. The conjugation of chemical drugs, if spaced properly (≥ 5 nt between each drug) will not promote aggregation of the nanoparticle. Temporary aggregation of the RNA 4WJ may be seen if buffers have a high Mg^{2+} concentration (>10 mM). The aggregation can be broken back to individual nanoparticles by removing Mg^{2+} via EDTA addition or buffer dialysis.
6. Conjugated chemotherapeutics are cleavable by esterase or hydrolysis of the ester allowing for stable conjugation and controlled release in the tumor environment. As shown in **Fig. 9e-f**, drug release has a half-life of ~8 hours in FBS serum that mimics *in vivo* circulation and aligns well with the time it takes for RNA nanoparticles to reach the tumor environment.^{34,40,209} Additionally, upon cleaving of the ester linker, the chemotherapeutic returns to its original structure and function demonstrating strong tumor inhibition,^{34,39-41} while improving upon the safety profile of the otherwise toxic chemotherapeutic.^{39,42} Due to the water sensitivity of the ester linker, it is important to store the 4WJ-drug conjugate at $-20^{\circ}C$ for short-term storage (> 6 months) and at $-80^{\circ}C$ for long-term storage. This will prevent hydrolysis of the ester linker and ensure the RNA nanoparticle retains its chemotherapeutic conjugates long-term. The high thermostability of the RNA 4WJ allows for storage in water or various buffers without concern of RNA nanoparticle dissociation. In general, though esters are sensitive to changes in pH and elevated temperatures. Therefore, storage buffers should maintain a neutral pH. Thermostability studies on temperature gradient gel electrophoresis did not show immediate paclitaxel release from the RNA 4WJ at $80^{\circ}C$ during the 1 hour it took to run the gel,³⁹ suggesting overall stability of the drug conjugate.
7. Inclusion of targeting RNA aptamers; such as EGFR,³⁹ EpCAM,⁴¹ or PSMA²⁹; on the RNA nanoparticle results in specific cellular uptake by cells expressing the respective receptor (**Fig. 10 a, e**). It is expected that flow cytometry and confocal microscopy will demonstrate selective uptake of targeted nanoparticles over control nanoparticles without the aptamer. As a result of cellular uptake of 4WJ-drug conjugates, prodrugs are cleaved in the endosome and drugs released into the cytoplasm. Cell viability assays demonstrate the controlled inhibition of colorectal cell proliferation by SN38 delivery (**Fig. 10b**) and TNBC cell proliferation by PTX delivery (**Fig. 10f**). Finally, when delivered intravenously into mice harboring tumors, RNA nanoparticles demonstrate significant tumor growth inhibition compared to controls (**Fig. c, g**), with tumors showing significant differences in weight (**Fig. 10d, h**).

[H1] AUTHOR CONTRIBUTIONS

P.G. and D.W.B. conceived and led the project. D.W.B., K.J., Y.T. designed and conducted the experiments and co-wrote the manuscript with P.G.

[H1] COMPETING FINANCIAL INTERESTS

P.G. is the licenser of Oxford Nanopore Technologies; co-founder of ExonanoRNA, LLC; and the consultant of RNA Nanobiotics.

[H1] DATA AVAILABILITY

Data that support this protocol can be found in their original publications^{34,39-41,61} and are included as Supplementary Data files.

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TABLES

Table 1: Sequences of RNA nanoparticles.	
RNA strand	Sequence (5'-3')
4WJ _A	5'-uuAGGuAAAGccAccuGcAGGuGcuAccGAuGuAAuucAA-3'
4WJ _B	5'-uuGAAuuAcAucGGuAGcAcGGGcuGuGcGAGGcuGAACAG-3'
4WJ _C -EGFR _{apt}	5'-cuGuucAGccucGcAcAGccAGcAcGcAccuGAAuAGGuGccuuAGuAAcGuGcuuuGAuGucGAuucGAcAGGAGGc-3'
4WJ _C	5'-cuGuucAGccucGcAcAGccAGcAcGcAccuGAAuAGG-3'
4WJ _D	5'-ccuAuucAGGuGcGuGcuGGGcuGcAGGuGGcuuuAccuAA-3'
4WJ _A -6-ALK	5'-uuAGGuAAAGccAccuGcAGGuGcuAccGAuGuAAuucAA-3'
4WJ _B -6-ALK	5'-uuGAAuuAcAucGGuAGcAcGGGcuGuGcGAGGcuGAACAG-3'
4WJ _C -6-ALK-EGFR _{apt}	5'-cuGuucAGccucGcAcAGccAGcAcGcAccuGAAuAGGuGccuuAGuAAcGuGcuuuGAuGucGAuucGAcAGG AGGc-3'
4WJ _C -6-ALK	5'-cuGuucAGccucGcAcAGccAGcAcGcAccuGAAuAGG-3'
4WJ _D -6-ALK	5'-ccuAuucAGGuGcGuGcuGGGcuGcAGGuGGcuuuAccuAA-3'
All C and U are 2'-fluoro modified (lowercase). All <u>5'</u> are 5'-Hexynyl modified, and all <u>C</u> and <u>U</u> are 2'-O-propargyl modified.	

FIGURE LEGENDS

Figure 1: Overview schematic of protocol to produce branched RNA nanoparticle conjugated with small molecule chemical drugs. Modified with permission from (Ref³⁹;). Copyright © 2020 Guo *et al.* under CC By 4.0 <https://creativecommons.org/licenses/by/4.0/>.

Figure 2: Workflow of protocol for generating chemical drug conjugated RNA nanoparticles.

Figure 3: Overview of ultra-stable 4WJ RNA nanoparticle for chemical drug conjugation. (A) Predicted structure of the 4WJ RNA nanoparticle (right) derived from the Phi29 pRNA-3WJ (left). (B) 12% (wt/vol) native PAGE in TBE showing assembly of the 4WJ RNA nanoparticle from 4 short RNA oligos showing high assembly yield (M: monomer, D: dimer, T: trimer controls). (C) Cryo-EM reconstruction of the 4WJ RNA nanoparticle matching predicted structures. (D) Dynamic

light scattering of the 4WJ RNA nanoparticle (red) compared to the 3WJ (blue), both showing homogenous products. **(E)** Thermostability of the 3WJ and 4WJ RNA nanoparticles without (left) and with (right) paclitaxel conjugation showing strong increased annealing temperature (T_a) of the 4WJ by SYBR Green II fluorescence measured using a thermocycler. Reproduced with permission from (Ref³⁹). Copyright © 2020 Guo *et al.* under CC By 4.0 <https://creativecommons.org/licenses/by/4.0/>.

Figure 4: Synthesis scheme of chemical drug modification into prodrugs via Steglich esterification. **(A)** Chemical structure of chemotherapeutic drugs used for esterification reaction, with the most reactive hydroxyl sites highlighted in red. **(B)** Chemical reaction scheme of esterification reaction of Camptothecin as a model.

Figure 5: Synthesis scheme of drug conjugation to the RNA oligo using copper chelated click chemistry. Shown here is the SN-38 prodrug conjugated RNA oligo with 6 alkyne modifications.

Figure 6: Combinational delivery of SN38/gemcitabine (GEM) chemotherapies achieved by 4WJ RNA nanoparticles. Dose-response matrix of 4WJ-GEM/SN38 and highest single agent (HSA) synergy map of 4WJ conjugated with GEM and SN38. Reproduced with permission from (Ref⁴¹). Copyright © 2023 Elsevier Ltd.

Figure 7: Characterization of prodrugs following Steglich esterification. **(A)** ^1H NMR (400 MHz) for paclitaxel. Circles show identifying groups to demonstrate paclitaxel or paclitaxel-azide. **(B)** Mass spectra of Paclitaxel-azide: ESI-MS calculated for $\text{C}_{53}\text{H}_{60}\text{N}_4\text{NaO}_{15}$ is 1015.3953 and observed mass is 1015.4167. **(C)** LC-MS data of the synthesized SN38- N_3 . Reproduced with permission from (Ref⁶¹). Copyright © 2018 Elsevier B.V.; from (Ref³⁴). Copyright © 2020 Elsevier B.V.; from (Ref⁴¹). Copyright © 2023 Elsevier Ltd.

Figure 8: Characterization of RNA oligos conjugated with chemotherapeutics. RNA conjugates analyzed by reverse-phase HPLC (left), 15% (wt/vol) urea denaturing PAGE in TBE (middle), and chemical drug solubility measured by absorbance at 354 nm (camptothecin) and 366 nm (SN38) (right) for **(A)** camptothecin (CPT), **(B)** paclitaxel (PTX), and **(C)** SN38. Reproduced with permission from (Ref³⁹). Copyright © 2020 Guo *et al.* under CC By 4.0 <https://creativecommons.org/licenses/by/4.0/>; from (Ref⁴⁰). Copyright © 2020 Piao *et al.* under CC By 4.0 <https://creativecommons.org/licenses/by/4.0/>; and from (Ref⁴¹). Copyright © 2023 Elsevier Ltd.

Figure 9: Assembly and characterization of 4WJ RNA nanoparticles conjugated with chemotherapeutics. **(A)** Polyacrylamide gel of 4WJ assembly with PTX and EGFR aptamer showing stepwise assembly, (1: 4WJ_A-6PTX, 2: 4WJ_{AB}-12PTX, 3: 4WJ_{ABC}-18PTX, 4: 4WJ-24PTX, 5: 4WJ, 6: 4WJ-24PTX, 7: 4WJ-24PTX-EGFR_{apt}; M: monomer, D: dimer, T: trimer). Size distribution of 4WJ and PTX **(B)** ($n=3$ presented as average and error bars as standard deviation) or SN38 **(C)** by dynamic light scattering. **(D)** Polyacrylamide gel analysis of RNA nanoparticle stability following time course serum digestion by FBS. Drug release from RNA nanoparticle over time of PTX from RNA 6WJ **(E)** and CPT from RNA 3WJ **(F)** ($n=3$ presented as average and error bars as standard deviation). **(G)** Thermostability of 4WJ-SN38 demonstrated by temperature

gradient gel electrophoresis (left) and SYBR Green II fluorescence measured by thermocycler (right). **(H)** Zeta potential distribution of 4WJ and 4WJ-SN38. Reproduced with permission from (Ref³⁹). Copyright © 2020 Guo *et al.* under CC By 4.0 <https://creativecommons.org/licenses/by/4.0/>; from (Ref⁴¹). Copyright © 2023 Elsevier Ltd.; from (Ref³⁴). Copyright © 2020 Elsevier B.V.; from (Ref⁴⁰). Copyright © 2020 Piao *et al.* under CC By 4.0 <https://creativecommons.org/licenses/by/4.0/>.

Figure 10: Efficacy of drug conjugated 4WJ nanoparticles for treatment of cancer. 4WJ-SN38-EpCAM (E) treatment of colorectal cancer and 4WJ-PTX-EGFR treatment of triple negative breast cancer by **(A, E)** cell targeting and internalization by confocal microscopy (scale bar: 100 μ m; Blue: nucleus, Green: cytoskeleton, Red: RNA nanoparticle), **(B, F)** cell proliferation inhibition assessed by MTT assay (n=3 presented as average and error bars as standard deviation), **(C, G)** tumor volume measured during treatment (n=5 presented as average and error bars as SEM for (C) or standard deviation for (G)), and **(D, H)** tumor images and tumor weights at the conclusion of treatment (n=5 presented as average and error bars as SEM for (D) or standard deviation for (H)). Reproduced with permission from (Ref⁴¹). Copyright © 2023 Elsevier Ltd.; from (Ref³⁹). Copyright © 2020 Guo *et al.* under CC By 4.0 <https://creativecommons.org/licenses/by/4.0/>.



















