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Efficient bioactive oligonucleotide-protein conjugation for cell-targeted cancer therapy

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Oligonucleotide-protein conjugates have important applications in biomedicine. Simple and efficient methods are described for the preparation of these conjugates. Specifically, we describe a new method in which a bifunctional linker is attached to thiol-oligonucleotide to generate a reactive intermediate that is used to link to the protein. Having similar conjugation efficacy compared with the classical method in which the bifunctional linker is attached first to the protein, this new approach produces significantly more active conjugates with higher batch to batch reproducibility. In a second approach, direct conjugation is proposed using oligonucleotides carrying carboxyl groups. These methodologies have been applied to prepare nanoconjugates of an engineered nanoparticle protein carrying a T22 peptide with affinity for the CXCR4 chemokine receptor and oligomers of the antiproliferative nucleotide 2′-deoxy-5-fluorouridine in a very efficient way. The protocols have potential uses for the functionalization of proteins, amino-containing polymers or amino-lipids in order to produce complex therapeutic nucleic acid delivery systems.

Oligonucleotide-protein conjugates hold great promise for studying DNA-protein interactions while being versatile molecular tools for biotechnology and material science.[1–3] To this end, DNA-protein conjugates have been used to fabricate arrangements of proteins using the specificity of base-pairing of nucleic acids or to immobilize proteins to surfaces in the fabrication of protein arrays or other supramolecular devices.[3–5] Additionally, several immunological bioassays are based on the use of these chimeras.[6]

The attachment of oligonucleotides to proteins or other polymers have shown to enhance nucleic acids delivery in gene inhibition strategies.[7,8] In this sense, polymer-DNA drug conjugates are one of the approaches to modify solubility, biological activity and stability of these drugs.[9]

Several methods have been proposed to link synthetic oligonucleotides to proteins using both non-covalent and covalent approaches. The reversible non-covalent strategy (e.g. biotin-streptavidin[10] or nickel-histidine[11]) produce poorly stable conjugates with difficult handling.[7,12] A wide variety of chemistries for covalent conjugation have been reported.[1–4] Some of them are based on the modification at a predetermined site of the oligonucleotides with reactive groups that directly react with the amino groups of lysines or the thiol groups of cysteines obtaining amide or disulfide bonds respectively.[13,14] More complex strategies have also been reported using post-transcriptional modification of the protein or involvement of a catalytic or reactive protein domain.[15] Since biomolecules are often present at low concentrations, the conjugation depends on the reaction rate and the stability of the reacting groups. More efficient results were obtained using bioorthogonal approaches in which the reactive groups do not modify the activity of the biomolecules. Specifically, bioorthogonal methods such as copper-catalyzed azide-alkyne [3 + 2] cycloaddition[12] or oxime and hydrazone ligation have been proposed.[16] In addition, several bifunctional cross-linkers have been also described to produce protein conjugates.[17,18] The most frequently used bifunctional linkers carry maleimide and N-hydroxysuccinimidyl ester groups. These compounds are first coupled with the protein to produce maleimide-protein intermediates. Then, after removal of the excess of cross-linker, the maleimide group is reacted with a thiol modified oligonucleotide. However, inversion of the reactive groups has also been described in which amino modified DNA reacts with the biofunctional linker producing a maleimide-oligonucleotide that finally reacts with the free thiol of cysteines of the protein.[19]

Multifunctional proteins have emerged as powerful engineered chimeric proteins that incorporate several selected domains. Among them, protein-based nanocarriers based on the concept of virus-mimetic vehicles have been explored for

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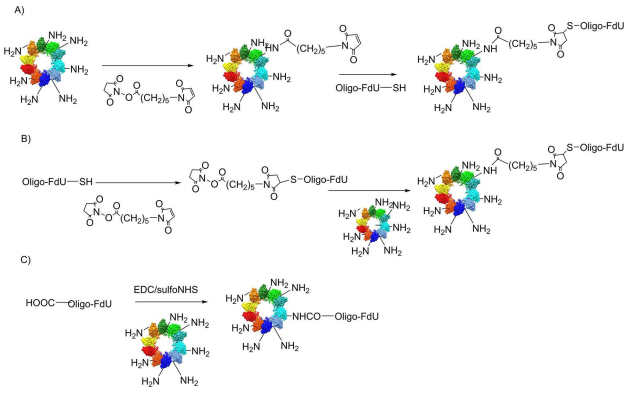
drug delivery.[20] Some of us have developed protein nanoparticles for targeted drug delivery to tumor cells that overexpress the chemokine receptor CXCR4. This receptor is associated with metastatic progression in several tumor types, including colorectal cancer and in human immunodeficiency virus infection.[21–23] This carrier incorporates the T22 peptide, a known ligand of CXCR4, which internalizes into CXCR4+ cells via specific receptor endocytosis.[24,25] This engineered self-assembling protein incorporates a green fluorescent protein and a histidine tag.[26] The resulting T22-GFP-H6 protein nanoparticle has been reported to be an excellent drug carrier in cell culture and for *in vivo* targeting to cells that overexpress the CXCR4 receptor in metastatic colorectal cancer mouse models.[27]

5-Fluoro-2′-deoxyuridine (FdU) is a cytotoxic drug known for its high antitumor activity against cancer.[28] Short oligomers carrying FdU have been described as prodrugs producing FdU monophosphate inside the cells.[29–32] FdU monophosphate is the active form of FdU as it can be converted to FdU triphosphate and induce the synthesis of mutated DNA that provoke cell death. In addition FdU monophosphate is an inhibitor of thymidine synthase producing thymine-less cell death. The use of short FdU oligomers for cancer treatment has been described in the bibliography[29,32] but delivery of the antiproliferative oligomers remains an unsolved problem.[30,31] Our present interest is to functionalize this T22-GFP-H6 protein nanoparticle with nucleic acid drugs for cancer treatment. To this end, we have selected the functionalization of T22-GFP-H6 protein with oligonucleotides carrying several units of FdU using the method based on the thiol-maleimide reaction. We have demonstrated that this conjugate showed antitumor but specifically antimetastatic activity in a CXCR4+ metastatic colorectal cancer model.[33]

The classical method consists of two steps (Scheme 1A, and supporting information S2-S8). First, T22-GFP-H6 protein is treated with an excess of a bifunctional cross-linker 6-Maleimidohexanoic acid N-hydroxysuccinimide ester (EMCS) and the resulting maleimide functionalized protein is reacted with the 3′-thiol-oligonucleotide. This two-step protocol was efficient but time-consuming as the excess of the EMCS has to be removed from the protein by dialysis and the maleimide group attached to the protein can slowly react with amino groups of Lys that contains the protein. This really was inconvenient because we observed in some cases a low functionalization of the protein due to the decrease of available maleimide groups to react with the 3′-thiol-oligonucleotide and low batch to batch reproducibility. In order to solve these problems we studied the reversal of the order of the reactants by reacting first the bifunctional crosslinker with the thiol-oligonucleotide and then the resulting oligonucleotide carrying the N-hydroxysuccinimidyl ester was used for the functionalization of the protein (Scheme 1B and supporting information S8-S10).



Scheme 1. Representation of the reactions involved in the preparation of oligonucleotide-FdU protein conjugates. (A) The classical method involving the addition of the maleimide group to the protein and subsequent Michael addition of thiol-oligonucleotide. (B) Inverted one-pot addition method in which the 3′-thiol-oligonucleotide reacts with the maleimide group of the linker and then the oligonucleotide carrying the N-hydroxysuccinyl ester group reacts with the protein. (C) Direct method in which the 5-carboxy-oligonucleotide is activated and then conjugated with the protein nanoparticle. Nanoparticle in-silico representation modified from (34). Reprinted by permission of John Wiley & Sons, Inc.

Additionally, the formation of FdU-oligonucleotide-protein conjugates was also studied via direct amide formation using FdU oligomers carrying 5′-carboxyl groups (Scheme 1C and supporting information S10–S12). This approach avoids the use of bifunctional linkers.

In the present communication we show that the new approaches B and C are feasible, simpler and able to produce the oligonucleotide-protein conjugates with antiproliferative activity in one-pot reaction with equal or slightly higher efficiency. Additionally, these methods avoid the potential cross-linking of the protein as it was observed in the first method and, therefore, the conjugation rate could be kept constant between the different conjugate batches. However, although apparently similar product is obtained by all methodologies in terms of nanoconjugate size and drug payload, completely different functional performance is observed for the nanoconjugates; showing that the used conjugation methodology exerts high impact in resulting nanoconjugates functionality even though showing similar physicochemical properties.

T22-GFP-H6-$(FdU)_5$ nanoconjugates were generated by covalent binding of the T22-GFP-H6 nanoparticles and oligomers carrying five units of FdU (5′-$(FdU)_5$-hexaethyleneglycol-thiol-3′). For comparison purposes the classical approach (Scheme 1A) was also performed (see supporting information, S2–S8). The optimal conditions for the classical two-step approach are the use of 5 molar excess of the linker with respect to the protein followed by an overnight treatment with 5 molar excess of thiol-oligonucleotide. These conditions generate conjugates with an average number of around 3 oligonucleotides per protein that corresponds to 15 molecules of the FdU drug (Table 1). These oligonucleotide/protein ratios are considered optimal without compromising the functionality of the protein (binding to the CXCR4 receptor and internalization).[33] Unfortunately, using this protocol, we observed batch to batch differences in terms of FdU protein loading. To solve this problem, we studied the excess of the linker in the coupling reaction to the protein nanoparticle. The results, confirmed by gel electrophoresis, indicate that when we increase the linker excess, more bands corresponding to cross-

Table 1. Functionalization, size and antiproliferative activity (MTT assay) of the protein conjugates carrying FdU oligomers prepared in this work.

Conjugates	Method	Conditions	Units of oligo-drug /protein[a]	Size by DLS	MTT (IC_{50}) HeLa
T22-GFP-H6-FdU	Classical two-step protocol	1 mg/ml, ×5 drug excess	3 FdU/protein	11–14 nm	16.22 nM
T22-GFP-H6-FdU	Inverted one-pot addition	2 mg/ml, ×5 drug excess	3 FdU/protein	11–12 nm	2.08 nM
T22-GFP-H6-FdU	Direct -COOH coupling	2 mg/ml, ×5 drug excess	1.2 FdU/protein	9.5–13 nm	2.84 nM
BSA-FdU	Inverted one-pot addition	2 mg/ml, x 5 drug excess	3 FdU/protein	7.4 nm	n.d.
T22-GFP-H6-FdU	Inverted one-pot addition	2 mg/ml, ×50 drug excess	5.9 FdU/protein	11–12 nm	0.43 nM
T22-GFP-H6-FdU	Direct -COOH coupling	2 mg/ml, ×50 drug excess	4.9 FdU/protein	9.5–13 nm	1.08 nM

n.d.: not determined. [a]Average number of oligonucleotide units per protein measured by UV spectroscopy

linked nanoparticles trapped into the gel well are observed. Consequently, fewer operative nanoparticles are obtained that may affect the subsequent FdU loading (supporting information Figure S5). The potential side reaction of the amino groups of lysines to maleimido groups is in agreement with the observed instability of maleimido lysine-rich peptides described by Eritja et al.[35]

Based on the selective reactivity of thiol-oligonucleotides for the maleimido function[35] we decided to study the reversal of the order of the reactants (scheme 1B). In a first trial the thiol-oligonucleotide was mixed with ECMS in a 1:1 molar ratio and after 10 min of incubation time at room temperature the reaction mixture was added to the protein T22-GFP-H6 protein obtaining the desired protein-oligonucleotide conjugate. To our knowledge the selective reaction of thiol-oligonucleotides to maleimido groups in the presence of the *N*-hydroxysuccinimidyl ester has not been reported. Next, we optimized the one-pot inverted addition method in which the thiol-oligonucleotide is reacted first with equimolar amounts of the EMCS linker (for 10 min at room temperature) and then the mixture was added over the T22-GFP-H6 nanoparticles solution at a 1:5 (nanoparticles: oligo-FdU) molar ratio for 2 h at room temperature followed by an overnight incubation at 4 °C. The second step implies the amide formation by the activated ester of the linker functionalized oligo-FdU and the amino groups of the external Lys of the T22-GFP-H6. Following this method we obtained an average functionalization of up to 3 molecules of oligo-FdU per protein depending on the conditions, which corresponds to 15 molecules of FdU (see supporting information, S8–S10). Although the efficiency is similar using both methods, the second is faster as no dialysis is required to remove the linker from the oligo-FdU. Most importantly, we did not observe any cross-linking between different proteins (Figure S10) as we saw in the classical method (Figure S5) even at higher concentration of the linker or the protein (2 mg/ml), which is probably due to the reaction of lysines to a maleimido group. The best results in the one-pot inverted method were obtained performing an overnight conjugation at room temperature and at 2 mg/ml protein concentration (Table 1). The analysis by mass spectrometry of the dialyzed solutions after the coupling of the thiol-oligonucleotide showed the presence of the oligonucleotide-carboxylic acid coming from the hydrolysis of the N-hydroxysuccinimidyl ester. This compound was expected because of the use of aqueous media. Even with the potential hydrolysis of the N-hydroxysuccinimide ester during the thiol-maleimido reaction the yields are good if one considers that using a 5 times excess of thiol-oligonucleotide, approx. 3 molecules of oligonucleotide are attached to the protein. This suggests that the thiol group reacted mainly with the maleimide group of the bifunctional linker although we cannot discard some side reaction between thiol group and the active ester to yield a thioester oligonucleotide dimer that slowly hydrolyze to the oligonucleotide-carboxylic acid.

The inverted one-pot method was extended to produce conjugates with larger proteins. We choose BSA (Bovine Serum Albumin Fraction V, Roche). BSA has a molecular weight of ~68 KDa being twice the size the monomeric T22-GFP-H6 protein. BSA has 59 lysine residues, of which 30–35 have exposed primary amines that can react with the bifunctional cross-linker. For comparison, T22-GFP-H6 contains 23 Lys residues, all of them exposed. We have used the optimized inverted addition protocol to produce BSA-$(FdU)_5$ conjugates obtaining an average of 3 molecules of oligo-$(FdU)_5$ per protein (supporting information, S9, S10). It is worth to mention that in the case of BSA in spite of being a bigger protein than T22-GFP-H6 the DLS data indicate a smaller size (7.4 nm) compared with T22-GFP-H6. This is because T22-GFP-H6 self-assemble to produce a larger protein nanoparticle.[33,34]

The key step in the formation of the conjugates is the production of pure, active and sufficient activated products to conjugate with the protein. Next, we thought it was worth preparing an activated oligo-FdU without using bifunctional linkers. For that, conjugates were also obtained via direct coupling of activated COOH-oligo-FdU and the protein nanoparticle (Scheme 1C). A carboxylic group was added to the 5'-end of the oligo-FdU, then it was activated with sulfo-NHS (*N*-hydroxysulfosuccinimide) and EDC (*N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide) and, finally, it was allowed to react with the protein. Moderate efficacy (1.2 oligo-FdU) was obtained using the 5–10 molar excess (see supporting information, S10–S12).

Optimization of the reaction was accomplished using 50-fold excess of activated oligonucleotide followed by an overnight reaction with the protein at 2 mg/ml. Using these conditions we obtained 4.9 FdU oligonucleotide units per protein nanoparticle using the direct method (see supporting information). Similarly, using the same conditions, in the inverted one-pot addition, we obtained 5.9 FdU oligonucleotide units that was the highest ratio of oligo-FdU coupled to the nanoparticle protein. T22-GFP-H6-FdU conjugates were used for

blocking CXCR4+ metastatic colon cancer stem-cells.[33] Using these conditions, concentrated conjugates were obtained that were highly recommended for clinical testing as we reduced the volume required for intravenous administration.

Mass spectrometry of all conjugates confirmed the addition of oligo-FdU to the proteins (supporting information, Figures S2, S6-S9). Figure 1A shows the spectrum of T22-GFP-H6 nanoparticle and T22-GFP-H6-FdU conjugate prepared using inverted one-pot addition.

The volume size distribution of T22-GFP-H6 nanoparticles and T22-GFP-H6-FdU conjugates was also measured by Dynamic Light Scattering (Table 1 and Figure 1B). No significant particle size differences were observed.

Antiproliferative activity of FdU-protein conjugates was measured by MTT assays in CXCR4 overexpressing (CXCR4+) HeLa cells (see Figure 1C and supporting information S5). The more active FdU conjugate was the T22-GFP-H6-FdU nanoparticle conjugate prepared by inverted one-pot method and is in accordance with the higher FdU loading (Table 1). At 5 molar excess of drug, the conjugate prepared by inverted one-pot addition has 8 times higher antiproliferative activity than the classical two-step protocol, although both have the same FdU loading. This indicates that the new method is more efficient. In the direct method, the antiproliferative effect is more than 5 times higher than the classic method, although only 1.2 of oligo-FdU is conjugated. In contrast, direct exposure of HeLa cells to unconjugated oligo-FdU dramatically reduces the observed antitumor activity (IC_{50} = 275 nM, Figure 1H, reference [33]) since this molecule could not be concentrated in the cell cytosol because it does not use receptor-mediated endocytosis to enter the cell.

When we increased the excess of the oligo-FdU, the antiproliferative activity is slightly better in inverted one-pot addition method rather than the direct COOH coupling because more oligo-FdU is conjugated to the nanoparticle protein. Likewise, dose-response viability of the conjugate T22-GFP-H6-FdU using direct method was similar and follows the same

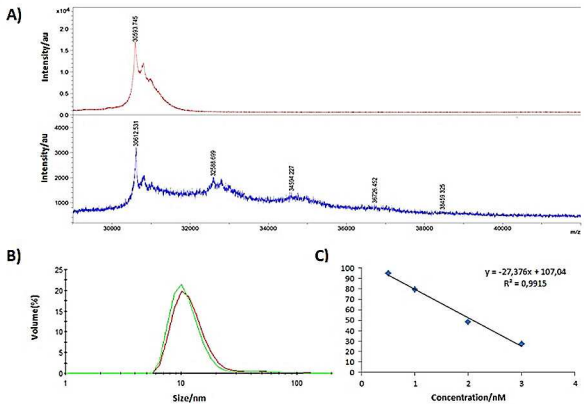


Figure 1. A). Mass spectrometry of the initial T22-GFP-H6 protein (in red) and the T22-GFP-H6-FdU conjugate (in blue). B) DLS of the T22-GFP-H6 protein (in green) and the T22-GFP-H6-FdU conjugate (in red) using inverted one-pot reaction. C) Dose-response representation of CXCR4+ HeLa cells exposed to different concentration of T22-GFP-H6-FdU conjugates for 48 h, analyzed by MTT viability assay.

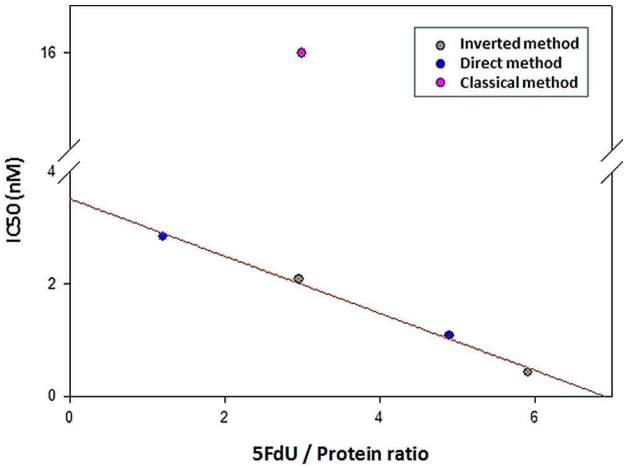
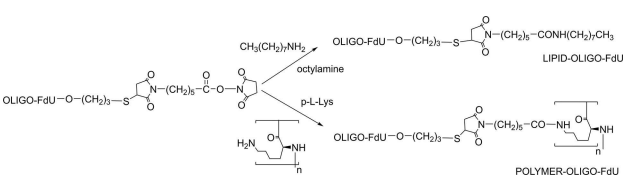


Figure 2. Functional characterization of nanoconjugates. T22-GFP-H6-FdU payload-response trend line representation. Antitumor IC50 concentration of each sample was calculated by dose-response curves in MTT cell viability assays upon exposure over CXCR4+ HeLa cells for 48 h. Grey dots represent samples generated by inverted one-pot addition, blue dots represent samples generated by direct-COOH coupling and purple dots represent samples generated by classical two-step protocol. Note that sample from classical two-step protocol doesn't fit in linear regression line.

lineal pattern as the inverted method. In both methods, the IC_{50} is directly proportional to the oligo-FdU conjugated, generating nanoconjugates with similar properties (Figure 2). However, in the classical method, the inhibitory properties not only depend on the FdU payload but also other potential factors such as the proportion of the cross-linking generated during the preparation of the nanoconjugate that may negatively affect their functionality.

We have extended this knowledge to the preparation of conjugates carrying oligonucleotide-poly-L-Lysine conjugates and oligonucleotide-lipid conjugate (Scheme 2, supporting information S12–S17). Poly-L-Lys is a hydrophilic cationic molecule with a high number of potential amino reacting groups.[36] We employed poly-Lys, average molecular weight 4200 with around 20–35 available Lys residues. Octylamine was used as a lipid molecule and contains a single amine.

The conjugation of oligonucleotides to cationic polymers such as poly(L-Lys) has been described to enhance oligonucleotide delivery to cells,[37] to stabilize duplex and triplex structures[38] and to allow the introduction of multiple labels.[39] However, there are few strategies to prepare poly-L-Lys-conjugated oligonucleotides.[37–40] These methods require several steps and are time-consuming procedures. Here we have



Scheme 2. Schematic representation for the preparation of lipid-oligo-FdU conjugates and polymer-oligoFdU conjugates using the inverted addition method.

prepared the T5-thiol and $(FdU)_5$-hexaethyleneglycol-thiol-3′ and applied the one-pot inverted addition of the bifunctional linker to generate poly-(L-Lys)-oligonucleotide conjugates. Two different conjugates were obtained, namely T_5-poly-L-Lys and $(FdU)_5$-poly-L-Lys (see supporting information). The mass spectra analysis revealed several sets of signals corresponding to multiple oligonucleotides attached to poly-L-Lys molecules (Figures S14-S16, Table S1). However, the major set of signals corresponds to the 1:1 oligonucleotide-poly-L-Lys conjugate.

Next, we applied the protocols for the synthesis of a simple model oligonucleotide-lipid conjugate.[41] Lipids are also interesting molecules used to improve the delivery of the oligonucleotides. Here we tested our inverted addition methodology for the addition of n-octylamine, a hydrophobic molecule carrying one single amino group. In this case, we obtained the corresponding T_5-octyl and $(FU)_5$-octyl conjugates in good yield. The simplicity of the conjugates allowed the standard reversed-phase HPLC purification (Figure S21) and the major peak was isolated and characterized by mass spectrometry (supporting information, Figures S18–S19). The possibility of quantification of the final product allows us studying the impact on the yields of small changes in the conditions used in the first step. Going from 10 min to 30 min incubation time does not affect the yield, but using a 1:2 (thiol-DNA/EMCS) molar ratio the yield of conjugate was clearly reduced (see supporting information, S18–S19). This indicates that it is more critical to adjust the 1:1 molar ratio than the time of incubation of thiol-DNA with EMCS.

Conjugation of biomolecules offers the possibility of creating new entities with improved properties. For instance, conjugation of oligonucleotides to proteins allows the assembly of proteins for nanostructure arrangements, biomedical diagnostics or for an efficient delivery of the therapeutic nucleic acids.

Bifunctional linkers, although having appeared a long time ago, are now frequently used to generate new reacting groups for further coupling reactions. Several linkers with different reacting groups, length and hydrophobicity are now commercially available. Here, we explored the specific reactivity of biofunctional linkers to generate different oligonucleotide conjugates. In particular, we improved the coupling conditions compared to the classical method obtaining similar conjugates in less time; also, we demonstrated the versatility of the inverted one-pot addition method to modulate the number of nucleic acids derivatives attached to the proteins by changing the reaction conditions. In addition, we have demonstrated that the direct conjugation of the biomolecules is also possible in good yields without the use of the bifunctional linkers. This has been accomplished because it is possible to introduce several types of functional groups such as carboxyl groups at different positions of nucleic acids in a very efficient way.

In proteins, the most available reacting groups are the amino groups from Lys and, for this reason, are often used for the conjugation with other molecules or materials. For example, this approach has been used for the synthesis of the antibody-drug conjugates for cancer therapies. Similarly, our methods of preparing protein-nucleic acids drug conjugates rely on the reactivity of the lysines. We have used coupling conditions that provided the expected conjugates in a reproducible and efficient way obtaining similar drug/protein ratios. Importantly, we have demonstrated that not only the number but also the distribution of the drug is important in the efficacy of the protein conjugate. The order of the reacting bifunctional linker in the synthesis of the protein conjugates plays an important role in the production of nanostructures. The results confirmed that using the inverted-addition method, although we did no increase the number of conjugated oligo-FdU, the antiproliferative effect was around eight times higher compared to classical method. In addition, we observed a higher reproducibility of the different batches of the inverted-addition compared to classical method. Similar results were obtained using the direct method without using bifunctional linkers. Both approaches, inverted one-pot and direct methods, produce nanoparticle conjugates with antiproliferative properties directly proportional to the FdU payload. However, the classical method, although similar physical properties are obtained (size and payload), the resulting product is different regarding functionally and much less efficient.

The proposed methodologies have also been applied to generate oligonucleotide conjugates with lipids or poly-L-Lys, with different number of amino reactive groups, proving their extended applicability to different potential drug carriers. In particular, we can envision the use of these simple and useful methodologies to conjugate oligonucleotides to other molecules of interest such as carbohydrates, peptides or antibodies.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Oligonucleotide-protein conjugate · nanoparticle protein · 2′-deoxy-5-fluorouridine · polymers · lipids

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