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A FEASIBLE APPROACH TO EVALUATE THE RELATIVE REACTIVITY OF NHS-ESTER ACTIVATED GROUP WITH PRIMARY AMINE-DERIVATIZED DNA ANALOGUE AND NON-DERIVATIZED IMPURITY

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Abstract

Synthetic DNA analogues with improved stability are widely used in life science. The 3' and/or 5' equivalent terminuses are often derivatized by attaching an active group for further modification, but a certain amount of non-derivatized impurity often remains. It is important to know to what extent the impurity would influence further modification. The reaction of an NHS ester with primary amine is one of the most widely used options to modify DNA analogues. In this short communication, a 3'-(NH₂-biotin)-derivatized morpholino DNA analogue (MORF) was utilized as the model derivatized DNA analogue. Inclusion of a biotin concomitant with the primary amine at the 3'-terminus allows for the use of streptavidin to discriminate between the products from the derivatized MORF and non-derivatized MORF impurity. To detect the MORF reaction with NHS ester, S-acetyl NHS-MAG₃ was conjugated to the DNA analogue for labeling with ^{99m}Tc, a widely used nuclide in the clinic. It was found that the non-derivatized MORF also reacted with the S-acetyl NHS-MAG₃. Radiolabeling of the product yielded an equally high labeling efficiency. Nevertheless, streptavidin binding indicated that under the conditions of this investigation, the non-derivatized MORF was five times less reactive than the amine-derivatized MORF.

Keywords

Chelator; conjugation; radiolabeling; DNA analogues

INTRODUCTION

Synthetic DNA analogues are widely used in molecular biology or molecular medicine in place of the natural phosphodiester DNAs because of their enhanced in vivo stability.^[1–8] A probe, linker, or functional group is often attached to either the 3' or 5' equivalent terminus to inhibit cell growth, to effect cell killing, or to trace the presence or fate of the DNA analogue in living cells as well as in vivo in animals. Phosphorodiamidate morpholino oligomers (MORFs) are a family of synthetic DNA analogues. The chemical form of the 3'-equivalent terminus off the solid phase synthesis is a secondary amine. As with other DNA analogues, a primary amine, a carboxylate group, and other functional groups such as a biotin or a dye are often attached to one or both of the terminuses. The primary amine or the carboxylate group is often used for further conjugation with biologicals,^[8–11] novel probes, chelators, or radiolabeling precursors.^[10,12–15] Accordingly, any progress or knowledge about the modification chemistry would facilitate their application to biological investigations.

In our studies of tumor pretargeting using 3'-primary amine-derivatized MORFs,^[4,9,16–18] we observed some heat-sensitive or heat-unstable (labile) conjugates between MORF and chelators (p-isothiocyanate-benzyl-DTPA and S-acetyl NHS-MAG₃), which led to a low-labeling efficiency. We resolved the problem by pre-heating the conjugate to destroy and remove the labile attachment before radiolabeling, without carefully investigating the mechanism.^[12,14] Pre-heating continues to be used to avoid the interference in the current investigation.

Recently, we needed to determine whether the stable conjugate between an NHS-ester and a primary amine-derivatized MORF is exclusively formed via the primary amine in the presence of non-derivatized impurity. We conjugated a mixture of a primary amine-derivatized MORF and a native MORF with NHS-MAG₃ and radiolabeled them to trace the conjugates. In addition, a biotin was concomitantly incorporated in the primary amine-derivatized MORF (denoted as (NH₂-Biotin)-MORF). Streptavidin (SA) was used to discriminate between the conjugates of the derivatized MORF and the non-derivatized (or native) MORF. Presence of radiolabeled MORF not binding to SA would indicate that some MAG₃-MORF was formed from the non-derivatized MORF by side reactions instead of the reaction to the primary amine.

MATERIALS AND METHODS

A MORF and its complement (cMORF, also a phosphorodiamidate morpholino oligomer) were custom-synthesized by Gene-Tools (Philomath, OR). The MORF was also custom-derivatized by attaching a primary amineplus-biotin group (NH₂-Biotin) to the 3'-equivalent terminus. The MORF sequence was (5'-TCTTCTACTTCACA ACTA) and the terminal structures of the two MORFs are illustrated in Figure 1. The S-acetyl NHS-MAG₃ was

previously synthesized in house and confirmed by elemental analysis, proton nuclear magnetic resonance (NMR), and mass spectroscopy as earlier reported.^[12] The EZ™ Biotin Quantitation Kit and the streptavidin were purchased from Pierce (Thermo Fisher Scientific, Rockford, IL). The P-4 resin (Bio-Gel P-4 Gel, medium) was purchased from Bio-Rad Laboratories (Hercules, CA). The ⁹⁹Mo-^{99m}Tc generator was from Perkin Elmer Life Science Inc (Boston, MA). All other chemicals were reagent grade and were used without purification.

The MORF concentrations were determined by UV spectrophotometry using the molar absorbances provided by the manufacturer. Size exclusion (SE) HPLC was used to analyze radiolabeling as well as the interactions between MORF and cMORF and between biotin and streptavidin. The HPLC system included a Superdex™ 75 column (optimal separation range: 1×10^2 to 7×10^3 Da; Amersham Pharmacia Biotech, Piscataway, NJ), a UV and a radioactivity in-line detector run by Millennium software (Waters, Milford, MA). The eluent was 10% acetonitrile in 0.1 M NH₄Cl at a flow rate of 0.60 mL/minute. The radioactivity recovery was measured routinely and was always greater than 90%.

Measurement of (NH₂-Biotin) Group

The average number of biotins per MORF (i.e. the terminal modification efficiency of the MORF) was measured as described previously using the EZ™ Biotin Quantitation Kit and a spectrophotometer.^[19] The avidin-bound HABA (4'-hydroxyazobenzene-2-carboxylic acid) in the kit was orange in color, but became colorless after being replaced and released by biotin. The decrease in optical density was used to quantitate the amount of biotin.

As the HABA method could not distinguish whether a biotin was actually linked to the MORF, a SE-HPLC method was alternatively used to measure the average number of biotins per MORF by adding an excess amount of streptavidin (SA). The MORF with biotin is shifted to a retention time of higher molecular weight after binding to SA. An internal standard was used to quantitate the MORF not binding to SA. Specifically, a MORF sample (0.8 μg/μL in 0.1 M MES-0.15 M NaCl at pH 5.2) was mixed with a given amount of iodohippuric acid as an internal standard. It was run on HPLC with and without adding SA (5 μg/μL in H₂O). Both the MORF that was not treated with SA and the MORF treated with SA but without (NH₂-biotin) stayed at the original position. Three SA/MORF molar ratios (1, 2, and 3) were used to confirm that the SA was sufficient to shift all the biotinylated MORF. The absolute amount of the solution injected was not accurately controlled and the peak areas were normalized to the same level using the internal standard. The number of biotins per MORF was calculated as the shifted percentage of the original peak area of the MORF. After confirming all three molar ratios allowed for saturation of the biotin, the average and SD were calculated from the values measured at these three SA/MORF ratios.

MAG₃ Modification and Radiolabeling

As shown in Figure 2, the samples of native MORF, the (NH₂-biotin)-MORF, and a mixture of the two at a 1:1 MORF molar ratio were reacted with the S-acetyl NHS-MAG₃ following the protocol described previously.^[13] In the first step, approximately 1.5 mg of MORF was dissolved in 0.2 M, pH 8.0 HEPES to a concentration of 0.5 mg/mL. The solution was added

to a vial containing S-acetyl NHS-MAG₃ such that the MAG₃/MORF molar ratio was 20. After 2-hour incubation at room temperature, the reaction mixture was purified over a P4 column (0.7 × 20 cm) using 0.25 M NH₄OAc at pH 5.2 as eluent. The peak fractions with optical density (OD) value at 265 nm greater than 5 were pooled. Subsequently, another step was performed to dissociate the unstable MAG₃-MORF conjugates and remove the released MAG₃ chelator. Specifically, the MORF solution was mixed with a tartrate buffer (pH 9.2, 50 μg/μL Na₂Tartrate·2H₂O in a solution of 0.5 M Na₂HCO₃, 0.25 M NH₄OAc, and 0.175 M NH₃) and a fresh tin solution (10 μg/μL SnCl₂·2H₂O and 1 μg/μL NaAscorbate in 10 mM HCl). The volume ratios of MORF solution/tartrate buffer/tin solution were 15/5/1. The mixed solution was heated at 100°C for 20 minutes and purified again over a longer P4 column (1.0 × 50 cm), followed by pooling the peak fractions with OD values over 5 as the final stock conjugate solution.

Each MAG₃-conjugated MORF solution was labeled with ^{99m}Tc. Between 5 and 50 μL of ^{99m}Tc-pertechnetate generator eluate was added to a mixed solution of 30 μL of MAG₃-MORF solution (0.2-0.4 mg/mL MORF) in pH 5.2 NH₄OAc buffer, 10 μL of tartrate buffer, and 3 μL of 4 μg/μL SnCl₂·2 H₂O in ascorbate-HCl solution (1 μg/μL NaAscorbate in 10 mM HCl), followed by heating at 100°C for 20 minutes.

Shift of Radiolabeled MORF on SE HPLC by cMORF and Streptavidin

To confirm that the radioactivity peak faithfully represented the labeled MORFs, they were hybridized with cMORF. When the duplex formed, the labeled single-strand radioactivity peak shifted to the position of the duplex. The cMORF was in a great excess (at a cMORF/MORF molar ratio of ~ 55) as compared to the labeled MORF. Thus, the cMORF were more than sufficient to bind any of the MORF preserving the hybridization affinity.

The labeled MORFs were also reacted with SA such that any labeled MORF molecule that carried a biotin was shifted to the SA position or slightly to the left of it. An excessive dose resulting in a molar ratio of SA/MORF = 10:1 was used to assure complete binding of the biotinylated MORF.

RESULTS

Number of Biotins on (NH₂-Biotin)-MORF

The results from the detection by the EZ™ kit and from the HPLC “shifting” are shown in Figures 3(A) and (B). The OD value from the kit method decreases linearly with the increasing MORF added. The number of biotins per MORF from the kit method is calculated to be 0.68 by the formula of (slope*MW*volume)/(34000*MORF concentration). The value of lower than 1 indicates an incomplete terminal derivatization. This result is consistent with the results from the HPLC method as shown in Figure 3(B). Addition of streptavidin to (NH₂-biotin)-MORF at a molar ratio of 1:1 “shifts” most of the MORF peak to the left. Further addition of streptavidin has no additional effect, confirming the residual MORF lacks the (NH₂-biotin). The fraction of the MORF peak area reduced after addition of streptavidin was calculated to be 0.65 ± 0.01 based on the formula of [1 – residual peak

area_{n:1}]/[original peak area_{0:1}], ($n = 1, 2$ and 3). This value was also subsequently used to estimate the relative reactivity of the modified vs. the unmodified impurity.

Shift of Labeled MORFs on HPLC by Its Complement and SA

As shown in the top row of Figure 4, it is surprising that after MAG₃ modification, both the (NH₂-biotin)-MORF and the native MORF (top row) can be labeled with high efficiency (>95%). The fact that the native MORF can be labeled indicates the NHS-ester also reacts with some groups besides the primary amine. All of the labeled MORFs can hybridize with the cMORF (2nd row), indicating the overall integrity after conjugation and labeling.

The bottom row demonstrates the SA shifting results. For the (NH₂-biotin)-MORF sample containing 35% unmodified impurity (right panel), 5–10% is not shifted. It is reasonable that the native MORF labeled with ^{99m}Tc-MAG₃ cannot be shifted due to the lack of biotin (left panel). In the middle panel for the 1:1 mixture, 29.3% is not shiftable.

Theoretically, the reactivity of the non-derivatized MORF impurity with NHS-MAG₃ relative to the (NH₂-biotin)-derivatized MORF may be estimated directly from the right panel (the sample containing 35% unmodified impurity), but the 5–10% unshiftable residual is too small for an accurate estimation. For this reason, the 1:1 MORF molar mixture of the (NH₂-biotin)-MORF sample and the native MORF sample was included (Figure 4, middle panel), assuming the impurity was in an identical chemical form to the native MORF. The assumption was based on several corroborating observations. First, the impurity displays identical location on HPLC either with a label (Figure 4, 1st row) or without a label (Figure 3(B) vs. the HPLC trace of native MORF not presented). Second, labeled and unlabeled impurities exhibit identical binding to the cMORF (Figure 4, 2nd row). Third, neither impurity reacts with SA (Figure 4, 3rd row). Finally, personal communication with Dr Yongfu Li from Gene-Tools on the modification chemistry confirms this assumption. Based on the shiftable 70.7% in the 1:1 mixture, the relative reactivity of the modified to the unmodified MORF is estimated following the formula of $70.7\% = 65/(65 + 135/x)$, where the relative reactivity x is the number of unmodified MORF equivalent to one primary amine modified MORF in reactivity. The value of x is calculated to be 5.02. In other words, the reactivity of native MORF is five times weaker than the (NH₂-biotin)-MORF under the conditions of this investigation. Based on this relative reactivity, the predicted shiftable percentage for the (NH₂-biotin)-MORF sample is $90\% = 65/(65 + 35/5)$, in agreement with the observed 90–95% in the right panel of bottom row of Figure 4.

DISCUSSION

From inspection of the chemical structure in Figure 1, the reaction sites for the NHS-ester in the native MORF possibly include the 3'-secondary amine, the 5'-amide, and aromatic amines in the base residues. Those in the (NH₂-biotin)-MORF are the same except the 3'-secondary amine is converted to a primary amine. The primary amine is the most active and the secondary amine ranks next. The amide is not active enough to react with the NHS ester in water solution. Therefore, the MAG₃ conjugation to the native MORF can be attributed to the secondary amine and others (aromatic amines) as depicted in Figure 5, but we do not know their relative contributions. For the (NH₂-biotin)-MORF, we still do not know the

contribution of those aromatic amine sites, but we do know the four-fold increase in reactivity is due to the primary amine, because conversion of the native MORF to (NH₂-biotin)-MORF only involves attaching the primary amine group to the secondary amine (Figure 1). In addition, as shown in Figure 5, the contribution from the secondary amine should also be credited to the primary amine, because the two sequences (other possible sites) are identical. Thus, although the native MORF can be conjugated and provides an equally high labeling efficiency, the primary amine attached to the (NH₂-biotin)-MORF accounts for at least 80% of the entire MAG₃ conjugation. In reality, it may be closer to 90 or 95% as the secondary amine is more reactive than aromatic amines.

It is fortunate that in most cases, if there is some unmodified impurity (native MORF), the byproduct and the main product of the subsequent conjugations are equally useful and the difference is usually not visible. This short communication reveals an otherwise silent mechanism using a novel approach that utilizes radiolabeling, a DNA/cDNA shift analysis, and a streptavidin/biotin shift analysis. Although this communication provides an estimate of five-fold more reactivity for the primary amine, it emphasizes the novelty of this approach and underscores the potential impact where the existence of impurity can be a concern. For example, although we previously found minimal reaction of a nucleophile with the native MORF (15), the native MORF may not be used as a negative control for site-specific labeling using NHS-MAG₃ as the chelator. The radiolabeling is such highly effective that formation of a small amount of conjugate would readily provide an equally high labeling efficiency.

The relative reactivity results were from one conjugation of the native MORF, the modified MORF, and the mixture of both. Our study demonstrated the feasibility of using this approach to determine the reactivity of NHS-ester activated group with primary amine-derivatized DNA analogue, relative to the non-derivatized impurity. However, we did not fully explore the reaction kinetics of the on-MORF amines with NHS ester. Future studies can investigate multiple parameters. For example, repeats of the conjugation to achieve enhanced statistical significance would further validate the methodology. Studies separately measuring the reaction kinetics for the primary amine-derivatized MORF and the native MORF may alternatively provide a rate ratio that can be compared with the “5-fold more reactive” concluded herein. A study in which an increasing amount of NHS ester is used to consume all primary amines would also help to confirm the higher reactivity of the primary amine. Finally, experiments with varying reaction conditions, for example pH and temperature, would be interesting with regard to how the rate ratio would vary with the change in these parameters.

CONCLUSION

The current investigation reveals that the MORF without the primary amine derivatization can also react with the NHS-MAG₃ to form stable conjugates. However, under the conditions of this investigation, the reactivity of the non-derivatized MORF is five times lower than that of the primary amine-derivatized MORF. The results indicate NHS-ester essentially reacts with the primary amine, but if the byproduct from reacting at another site

is a concern, the percentage of the non-derivatized MORF impurity needs to be controlled within a certain range.

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FIGURE 1.
The structures of (NH₂-biotin)-MORF and native MORF.

FIGURE 2.

A scheme describing the MAG_3 conjugation to the MORFs and purification for $^{99\text{m}}\text{Tc}$ labeling.

FIGURE 3.

(A) The OD values of the reaction mixture of 15 μL of HABA-avidin with an increasing amount of $(\text{NH}_2\text{-biotin})\text{-MORF}$ ($0.798 \mu\text{g}/\mu\text{L}$). (B) The HPLC traces at UV 265 nm of the $(\text{NH}_2\text{-biotin})\text{-MORF}$ mixed with an internal standard of iodohippuric acid after adding an increasing amount of streptavidin (SA).

FIGURE 4.

The HPLC traces of native MORF (left panel), (NH₂-biotin)-MORF (right panel), and their 1:1 mixture (middle panel) after NHS-MAG₃ conjugation and radiolabeling (top row). Also shown are the peaks after addition of excess cMORF at a molar ratio of cMORF/MORF = 55 (second row) and excess SA at a molar ratio of SA/MORF = 10 (bottom row).

FIGURE 5.
The relative reactivity of native-MORF to that of (NH₂-biotin)-MORF.