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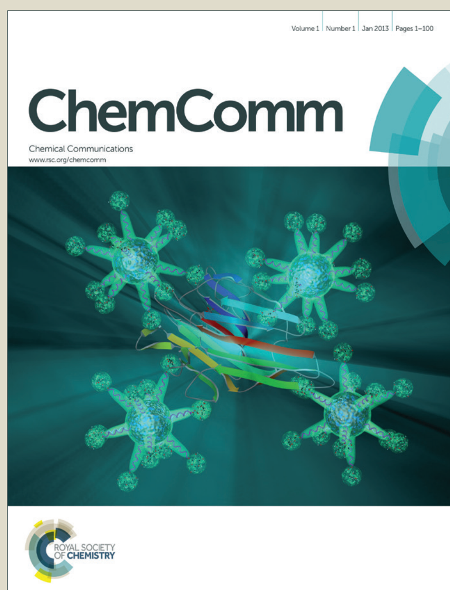

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Biotin-Conjugated *N*-Methylisatoic Anhydride, a Chemical Tool for Nucleic Acid Separation by Selective 2'-Hydroxyl Acylation of RNA.

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An isatoic anhydride derivative conjugated to a biotin and a disulfide linker was specifically designed for nucleic acids separation. Starting from a DNA/RNA mixture, a selective 2'-hydroxyl acylation of RNAs followed by capture with streptavidin-coated magnetic beads and cleavage of the disulfide led to elution of RNAs.

Since RNAs have been evidenced as playing fundamental roles in life such as coding or regulating important biological processes, they have been the focus of intense research efforts in order to establish their structure and functions. Advances in understanding RNA roles and structures have been enabled by altering RNA structures using simple chemical modifications, site-specific labeling or conjugation to other biomolecules. The manipulation of RNA to introduce labeling groups includes chemical RNA synthesis, post-synthetic RNA conjugation, enzymatic incorporation of tags to non-synthetic RNA strands, or chemical and enzymatic ligation of RNA strands.[1,2] Beside these manipulations of modified RNAs for nanotechnology applications, there is a great interest in the detection of natural RNAs as biomarkers for diagnostic applications or following treatment efficacy. For this detection to be efficient, the RNAs need to be extracted from a biological sample in an intact and pure form before being amplified.[3] A number of methods already exist for extraction and purification of RNAs.[4] Most of them are based on the biophysical properties of nucleic acids, and the most currently used method is the guanidinium-thiocyanate-phenol-extraction in which RNA and DNA partition respectively between the aqueous and organic phases.[5] This time-consuming and manual method requires to be carefully carried out, uses many centrifugation steps and mostly leaves DNA contamination. The use of DNAses remains very expensive and do not avoid a further denaturation step to inactivate DNAses before any amplification. Other methods based on chromatographic separation such as affinity or anion exchange chromatography can be used but lead to contaminated samples or are not easy to perform in most laboratories.[6] Traces of amplification inhibitors such as proteins or small molecules, cost, reagent toxicity and poor automation are also major drawbacks of these processes. Therefore, developing new methodologies for specific RNA extraction devoid of these disadvantages would be a significant step forward in the field of molecular biology including diagnostic applications. Among the chemical methods of biological RNA labeling, the 2'-OH selective modifications are of interest because the 2'-OH group constitutes a specific chemical signature of RNAs. Acylation of oligonucleotides using acetic anhydride has been described for a long time,[7] whereas the selective acetylation of the 2'-OH of mRNA was later described as giving high stability against ribonucleases.[8] Isatoic anhydride (IA) derivatives and their *N*-methylated counterparts *N*-Methyl Isatoic Anhydrides (NMIA) were recently described as RNAs 2'-OH selective acylation agents, and widely used for resolving secondary RNA structures using the SHAPE (Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension) technology.[9]

Owing to the selective reactivity of NMIA toward the 2'-OH group of ribose, we reasoned that a modified NMIA could be designed for the selective tagging of RNAs and used to discriminate DNA and RNA. The two nucleic acids could then be separated, purified before being used in different applications where their detection is required (Figure 1).

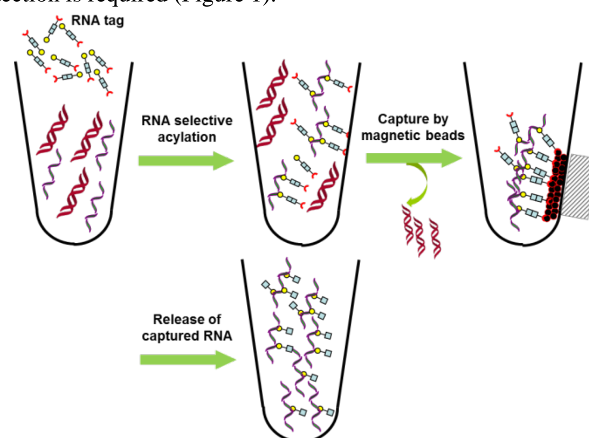


Figure 1. Principle of RNA/DNA separation

As a proof of concept, we designed a NMIA conjugated to a biotin through a linker containing a disulfide bond and a pegylated moiety which was used to improve water solubility (Figure 2). The biotin was used as a recognition group for a specific solid phase support capture of acylated RNA with streptavidin-coated magnetic beads. Captured RNA should be released from its support by the chemical cleavage of the disulfide bond. The RNA tag **7** was synthesized and first evaluated for the selective acylation of a 27-nt oligoribonucleotide *vs* oligodesoxy-nucleotide. The separation process was then performed with biological models of nucleic acids.

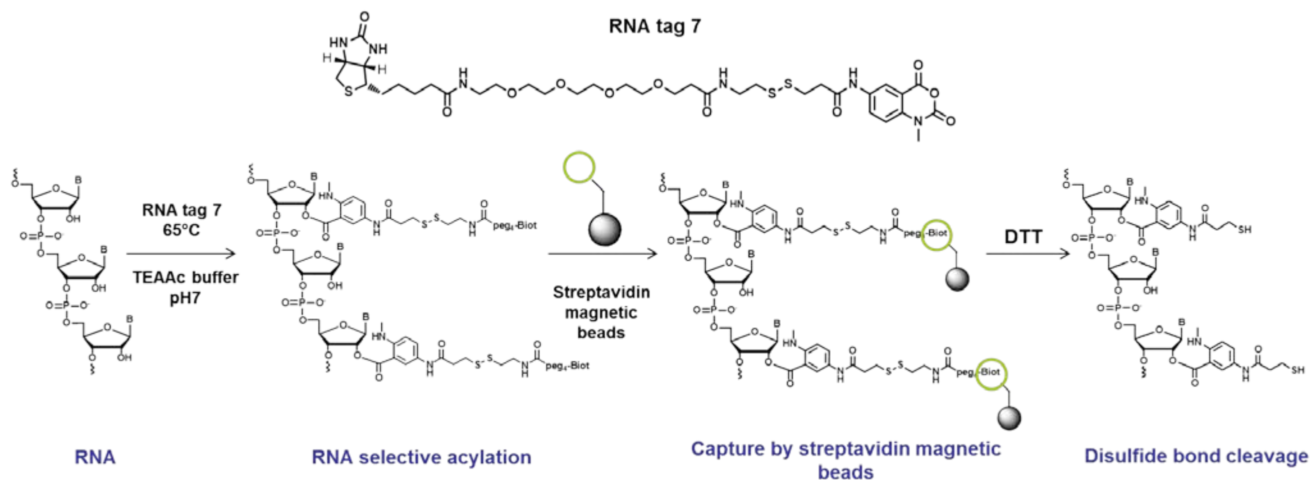
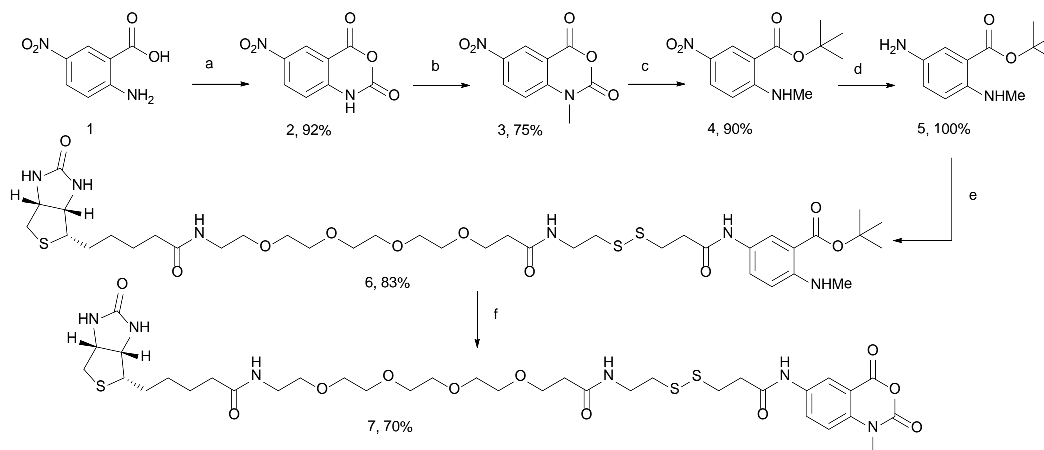


Figure 2. RNA purification process

An isatoic anhydride derivative bearing an amino group in position 6 was chosen to attach the biotin-peg$_4$-SS-COOH which is commercially available as an activated ester. However, due to the high reactivity of IA towards nucleophiles, it was necessary to use a precursor able to give the IA in the last step of the synthesis. According to the procedure developed by Sowell *et al.* for the generation of IA derivatives, we decided to use a *tert*-butyl amino ester as a precursor.[10] The synthesis of the *tert*-butyl 5-amino-2-(methylamino)benzoate **5** was performed from 2-amino-5-nitrobenzoic acid **1** through a four-step sequence (Scheme 1). 6-nitroisatoic anhydride was first generated by treatment with phosgene and subsequently *N*-methylated with iodomethane. The resulting *N*-methyl isatoic anhydride **3** was then opened with *t*-BuONa leading to the *tert*-butyl aminoester **4**. The quantitative reduction of the nitro group followed by coupling with Biot-peg$_4$-SS-CONHS led to the conjugated aminoester **6**. The latter was then reacted with phosgene to afford the IA **7** in a 36% overall yield.



Scheme 1. Synthesis of RNA tag 7.*

*Isolated yields. Reagents and conditions: a) $COCl_2$, dioxane, 0.5 h, rt. b) MeI, DIPEA, DMF, 1 h, rt. c) *t*-BuONa, DCM, 0.25 h, rt. d) H_2, Pd/C, AcOEt, 16 h, rt. e) Biot-peg$_4$-SS-CONHS, TEA, DCM, 1 h, rt. f) $COCl_2$, DCM, 0.25 h, rt.

The selective acylation of RNA with **7** was first examined using 27-nt RNA and DNA oligomers. Therefore, RNA or DNA oligomers were separately incubated with **7** in a DMSO/TEAAc buffer at 65°C for 1h. Tag excess was then removed after precipitation, and oligomers were subjected to an enzymatic digestion using a mixture of nuclease P1 and alkaline phosphatase. The resulting nucleosides were then analyzed by LC/MS and showed, for RNA oligomers, the presence of both non-modified and modified nucleosides. The ratio of tagged nucleosides was determined after enzymatic digestion by integrating peaks corresponding to the four free ribonucleosides and those corresponding to acylated nucleosides (Table 1). For the RNA oligomers, the ratio of tagged nucleosides *vs* free-tagged was found to be 7.5/92.5, while the same experiment, using DNA oligomers, led to a ratio of 0.7/99.3. These two experiments demonstrated the ability of

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7 to selectively acylate RNA *vs* DNA with a selectivity ratio of 10.7.

Table 1. Synthetic 27-nt RNA and 27-nt DNA tagging with **7**.

RNA tagging[a]		DNA tagging[a]		Tagged RNA/DNA ratio
Tagged nucleosides	Free nucleosides	Tagged nucleosides	Free nucleosides	
7.5±0.2	92.5±0.2	0.7±0.1	99.3±0.1	10.7±0,3

[a]ratio determined by LC/MS (n=2), see experimental data for details.

We then turned our attention to the separation of nucleic acids using biological models: HIV transcript for RNA and calf genomic DNA. First, the extraction process was studied with a HIV-transcript RNA (1500-nt) and a calf genomic DNA (20 kb) treated separately. The separation process was then applied to a mixture of both nucleic acids. For these experiments, nucleic acids were incubated with **7** (1.5 or 30 mM) and the tag excess was removed using magnetic silica particles. The biotinylated nucleic acids were then captured with streptavidin-coated magnetic beads and eluted after the disulfide cleavage with dithiotreitol (DTT). The quantities of RNA and DNA extracted by this method were determined by fluorescence. The selectivity of the capture was calculated as the RNA/DNA ratio obtained from these extraction yields (Table 2). When RNA and DNA were treated separately with **7** (1.5 mM), the extraction yields were evaluated to 27 and 0.5% respectively, leading to a selectivity ratio of 57. Increasing the concentration of **7** (30 mM) led to enhance both RNA and DNA extraction yields to 66 and 6% respectively. However, in this case a decreased selectivity ratio of 10.5, consistent with previous results on oligonucleotides at 30mM, was observed (Table 2). Although, a decrease in the extraction yield was obtained for the lower concentration, it led to a higher selectivity of RNA extraction. This selectivity associated to the quality of RNAs remains the key parameter for RNA-based diagnostic applications.

Table 2. Extraction of biological RNA or DNA.

NA	[7] (mM)	Extracted RNA yield (%)[a]	Extracted DNA yield (%)[a]	Extracted RNA/DNA yield ratio
RNA	1.5	27	-	57
DNA		-	0.5	
RNA	30	66	-	10.5
DNA		-	6	

[a]Determined using fluorescence spectroscopy, see experimental data for details.

The separation process was then applied to a 10/90 RNA/DNA mixture, considering the predominance of DNA in biological samples. For this experiment, **7** was used at the lower concentration of 1.5 mM in order to minimize the DNA side extraction. Using the same protocol, the RNA/DNA ratio after elution was calculated to 86/14, demonstrating thus the selectivity of the RNA acylation and capture in a mixture of nucleic acids (Table 3). Although we were able to reverse the initial *ratio* of nucleic acids, the residual presence of DNA was observed after the separation process. This result could be attributed to interactions between the two nucleic acids leading to the co-elution of DNA with the specifically captured RNA and not to a decrease in the selectivity of the acylation step in the mixture.

Table 3. Extraction of biological RNA/DNA mixture.

[7] (mM)	NA initial ratio (RNA/DNA)	NA final ratio (RNA/DNA)[a]
1.5	10/90	86/14±1.2

[a] Determined using fluorescence spectroscopy (n=4), see experimental data for details.

Owing to the low concentration of RNA obtained from biological samples, their amplification is a prerequisite to their efficient detection and analysis. Selective acylation of 2'-OH of RNAs has been used to stop the RT process and therefore analyze the whole RNA structure using the SHAPE technology.[9] For detection applications using amplification processes, only short sequences (50-200 nt) of RNAs are transcribed by the reverse transcriptase (RT), and the cDNA amplicons are then amplified. Since the acylation process occurs randomly on the RNA sequence, the reverse transcription of the extracted RNAs should be effective if the RT-target sequence remains unmodified after acylation.
In order to check the compatibility of our extraction protocol with the amplification techniques, we performed RT-PCR experiments. RNA HIV transcripts were reacted with 15mM of **7**, captured using streptavidin coated magnetic beads and eluted after disulfide cleavage by DTT. The extracted RNAs were then amplified by RT-PCR. Different experiments were performed by varying the number of tagged RNA copies per sample (50, 100 and 1000 copies, Figure 3b) and compared to amplification results using non-modified RNA (Figure 3a). The results show that HIV transcripts extracted after acylation can successfully be amplified, even with low RNA concentrations such as 50 copies and using a high concentration of tag (15 mM). Compared to non-acylated RNA, we can notice a slight decrease of fluorescence at all concentrations. This result can either be due to the acylation of RNA inside the amplification target sequence, leading to decrease the RNA concentration available for amplification, or to the quenching of fluorescence caused by the presence of anthranilate residues on the RNA sequence. We then checked the efficiency of the amplification by determination of the cycle thresholds (CT) for both acylated and non-acylated RNA (Figure 3C). No significant differences were observed for the two lower RNA template concentrations (50 and 100 copies), whereas at 1000 copies, only one additional cycle was necessary to reach the CT. Taken together, these results clearly demonstrate that RNA amplification remains consistent with this new extraction process, even at low concentrations of template, allowing its use for diagnostic applications.

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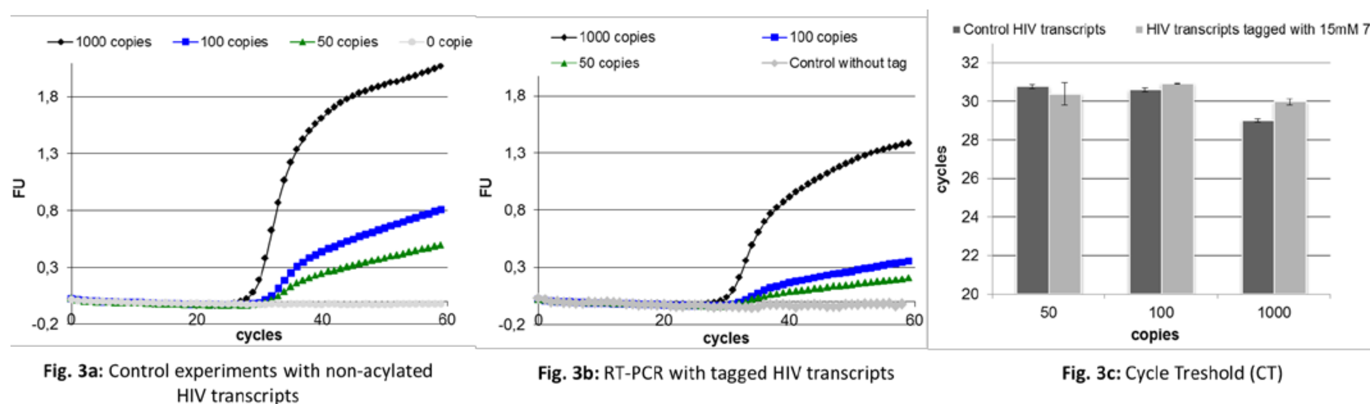

Fig. 3a: Control experiments with non-acylated HIV transcripts

Fig. 3b: RT-PCR with tagged HIV transcripts

Fig. 3c: Cycle Treshold (CT)

Figure 3. RT-PCR amplification of extracted HIV RNA

Conclusions

In summary, an isatoic anhydride derivative has been designed for the specific 2'-OH acylation of RNAs and has been successfully used in RNA selective capture from a mixture of biological RNA and DNA. We have demonstrated that this method can be used for extraction, purification and amplification of RNAs. The combination of the chemical selectivity of isatoic anhydrides toward RNA with their adequate functionalization could find other applications where selective RNA chemical labeling is needed.[11]

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† Electronic Supplementary Information (ESI) available: Detailed experimental procedures and characterization of new compounds. See DOI: 10.1039/c000000x/

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