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Hyphenation of a Deoxyribonuclease I immobilized enzyme reactor with liquid chromatography for the online stability evaluation of oligonucleotides

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ABSTRACT

The stability of antisense oligonucleotides (ONs) toward nucleases is a key aspect for their possible implementation as therapeutic agents. Typically, ON stability studies are performed off-line, where the ONs are incubated with nucleases in solution, followed by their analysis. The problematics of off-line processing render the detailed comparison of relative ON stability quite challenging. Therefore, the development of an online platform based on an immobilized enzyme reactor (IMER) coupled to liquid chromatography (LC) was developed as an alternative for improved ON stability testing. More in detail, Deoxyribonuclease I (DNase I) was immobilized on epoxy-silica particles of different pore sizes and packed into a column for the construction of an IMER. Subsequently, the hyphenation of the IMER with ion-pair chromatography (IPC) and ion-exchange chromatography (IEC) was evaluated, leading to the successful development of two online methodologies: IMER-IPC and IMER-IEC. More specifically, natural and modified DNA and RNA oligonucleotides were used for testing the performance of the methodologies. Both methodologies proved to be simple, automatable, fast and highly reproducible for the quantitative and qualitative evaluation of ON degradation. In addition, the extended IMER life time in combination with a more straightforward control of the reaction kinetics substantiate the applicability of the IMER-LC platform for ON stability tests and its implementation in routine and research laboratories.

1. Introduction

In the last two decades the therapeutic potential of antisense oligonucleotides (ONs) has been firmly recognized. These single-stranded short nucleic acid chains are designed to hybridize complementary sequences in DNA, pre-mRNA and mRNA, and in this way, block the expression of a target gene [1–3]. The main problems related with the development and implementation of therapeutic ONs include their intracellular delivery, transfection across the membrane and stability against nucleases [2,3]. In an attempt to improve upon the latter, many modifications on the phosphodiester backbone, the sugar or the base moiety have been described for increasing nuclease resistance [2,3]. For instance, the phosphorothioate modification is one of the simplest chemical modifications which can be carried out in the ON [3,4]. Hereby a non-bridging oxygen of the phosphorus is substituted by a sulfur atom. Phosphorothioates have demonstrated high resistance toward nucleases; however, their biggest drawback is the reduced ON hybridizing ability toward complementary strands [3,4]. Therefore, many other chemical modifications and their combinations have been considered in order to seek for an optimal balance between the hybridization ability of the ONs and their resistance toward nucleases [3–5].

Stability studies of ONs have been mainly performed in vitro [4,6–24]. For this purpose ONs are typically incubated with a specific nuclease, mixture of nucleases, biological fluids or with tissue homogenates, followed by their analysis in various ways. The initially developed analysis techniques required the usage of fluorescent or radiolabeled ONs in combination with polyacrylamide gel electrophoresis (PAGE) [6–8,10,14,15,25] or thin layer chromatography (TLC) [6]. However, those procedures were laborious, often lacking good sensitivity, while attaining accurate and precise quantitation results remained challenging. Afterwards, with the increasing use of capillary electrophoresis (CE) [23,24,26–28]

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and liquid chromatography (LC) [6,29], labeled ONs were no longer required. The usage of CE has proven to deliver a base to base resolution for ONs differing by one nucleotide in length. Moreover, the substitution of polyacrylamide filled capillaries by entangled polymer solutions drastically improved the reproducibility of the methodology and facilitated its implementation [27,30]. Nevertheless, the high ionic strength of the enzymatic incubation media, requires a desalting step of the sample for an adequate CE analysis [23,24,26,27,31], complicating in this way the sample manipulation procedure while affecting the analysis time and reproducibility of the methodology. In contrast, the greater robustness of LC, in addition to recent advances in the development of higher efficiency columns which are able to provide a high resolution separation of ONs in the range of 10-*mer* to 30-*mer* [29,30,32], made this technique the preferred candidate for ON stability studies in pharmaceutical environments [9,11,13,16,18,20–22]. Among the existing LC techniques, ion-pair chromatography (IPC) and ion-exchange chromatography (IEC) have proven to be suitable for this purpose [20,24,29,30,33]. Additionally, ON separations by IPC and IEC (with a polymeric support), do not occur exclusively based on the length of the ON, but also depend on the base composition [32]. Thus, discerning between two ON product fragments which have the same length but a significantly different base composition can be efficiently achieved using either of these two techniques [30]. Analytical procedures employed thus far for ON stability share in common the presence of enzymes in solution, for which the reaction is performed by batch incubation [4,6,9–11,13–16,18,19,25–27] or in an online way [34]. Incubation can be performed using either the enzyme present in solution or immobilized on a solid support. Enzyme immobilization offers several advantages when compared to enzymatic reactions carried out in solution. In addition to an easier separation of the enzyme from the reaction products and/or substrate, the immobilization of enzymes also confers them an increased stability toward mechanical stress, pH, heat, ionic strength and organic solvents [35–41]. Moreover, once packed into the columns to be used as immobilized enzyme reactors (IMERs), they can be employed in a continuous operation mode, reducing in this way the sample manipulation steps, increasing the reproducibility and allowing their reutilization; therefore, reducing costs and analysis time.

The immobilization of enzymes on solid supports has been widely studied and numerous immobilization methods and supports which are commonly employed have been reported in literature [35,36,38,40–43]. Several nucleases are commercially available, and a broad variety of combinations can be tested based on the type of substrate they hydrolyze (DNA or RNA), the type of nucleophilic attack (exonuclease and/or endonuclease), the nature of the hydrolytic products (mono or oligonucleotides) and the nature of the bond which is hydrolyzed [44,45]. Furthermore, factors such as their biological relevance, kinetic performance and cost, also need to be considered for their application in IMERs.

Deoxyribonuclease I (DNase I), is a 29.1 kDa endonuclease which cleaves the phosphodiester backbone of double and single stranded DNA, requiring divalent cations as cofactors [46,47]. The enzyme cleaves the P-O3′ bond of the DNA backbone, yielding 5′-phosphate oligonucleotides [47–49]. Moreover, DNase I has been used as a powerful footprinting agent and for DNA probing [50,51]. The immobilization of DNase I on monolithic supports [52,53], polymers [54] and magnetic particles [55] has been reported. Furthermore, the good catalytic performance of the immobilized endonuclease and its low cost, makes DNase I a good candidate for its implementation in IMERs.

In this study, we describe the construction of an IMER with bovine DNase I, and the evaluation of its hyphenation with IPC and IEC using natural and modified DNA and RNA ONs. Two online platforms (IMER-IPC and IMER-IEC) with potential applications in ON stability studies were successfully developed. To the best of our knowledge, the development of online IMER-LC methodologies for stability testing of ONs had not been reported yet, and could be of high relevance in the development of improved antisense ON therapies.

2. Experimental

2.1. Chemicals

Triethylamine (TEA), acetic acid, adenosine monophosphate (AMP), hydrochloric acid, chloroform, Tris, NaCl, acetone (HPLC grade), Na_2HPO_4, EDTA, $CaCl_2$, $MgCl_2$, glycine, (3-Glycidyloxypropyl) trimethoxysilane (GPTMS) and Deoxyribonuclease I (DNase I) from bovine pancreas (≥400 Kunitz units/mg of protein) were purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile (ACN) HPLC grade from Fischer Scientific (Loughborough, UK) and Milli-Q water (Milipore, Milfold, MA) were used. All the mobile phases were filtered through 0.22 μm nylon membrane filters (Grace Davison Discovery Sciences, Lokeren, Belgium). Homo-oligonucleotides of deoxythymidine 15-*mer* (T15), 20-*mer* (T20) and 30-*mer* (T30), deoxyadenosine 30-*mer* (A30), 12-*mer* ON (5′-GCA-CAC-CGT-CAG-3′) and a 41-*mer* ON (5′-GTT-GGA-TTA-AAC-AAC-CGT-TCC-CGT-CTC-TAT-CAG-CTT-AGT-GT-3′) all based on the phosphodiester backbone; 15-*mer* (5′-T*T*T*-T*T*T*-T*T*T*-T*T*T*-T*T*T*-3′) with a phosphorothioate backbone, and the 2′ O-methyl RNA 12-*mer* ON (5′-GCA-CAC-CGU-CAG-3′) were purchased from Eurogentec (Liege, Belgium). The lyophilized ONs were dissolved in an appropriate volume of Mili-Q water in order to obtain 100 μM stock solutions.

2.2. Instrumentation

An Agilent 1100 series HPLC composed of a binary pump and a single wavelength detector was employed for the delivery of the chromatographic mobile phases, while the enzymatic reaction buffer was delivered to the reactor by a HP 1050 quaternary pump (Agilent Technologies, Waldbronn, Germany). A CTO-20AC prominence HPLC column oven (Shimadzu, Kyoto, Japan) was used for controlling the temperature of the IMER.

2.3. Preparation of epoxy-functionalized silica

Nucleosil 5 μm spherical silica particles (1.2 g) of 300, 1000 and 4000 Angstrom from Macherey-Nagel (Duren, Germany) were used. The functionalization of the silica particles was carried out by suspending them in a 10% (v/v) GPTMS solution in acetone at room temperature for 2 h under intense stirring. Afterwards, the particles were filtered and thoroughly washed with acetone, followed by phosphate buffer (pH 7.0; 15 mM) containing 0.1 M NaCl and finally with phosphate buffer [56]. The particles were dried at 37 °C and then stored at 4 °C until the enzyme immobilization was carried out.

2.4. Immobilization of DNase I

Three mL of a 5 mg/mL solution of DNase I in phosphate buffer (pH 7.0; 15 mM) were mixed with 1 g of the functionalized silica particles and stirred at 3000 rpm for 30 min. Subsequently, the mixture was set in the fridge (4 °C) for 12 h. When the immobilization was completed, the enzyme modified silica was filtered and washed with phosphate buffer. Afterwards, deactivation of the unreacted epoxy groups was carried out by mixing the particles with a 0.2% (m/v) glycine solution in phosphate buffer and stirring for 2 h at

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