Immobilized enzyme reactors based on nucleoside phosphorylases and 2'-deoxyribosyltransferase for the in-flow synthesis of pharmaceutically relevant nucleoside analogues

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Abstract

In this work, a mono- and a bi-enzymatic analytical immobilized enzyme reactors (IMERs) were developed as prototypes for biosynthetic purposes and their performances in the in-flow synthesis of nucleoside analogues of pharmaceutical interest were evaluated. Two biocatalytic routes based on nucleoside 2'-deoxyribosyltransferase from *Lactobacillus reuteri* (*Lr*NDT) and uridine phosphorylase from *Clostridium perfrigens* (*Cp*UP)/purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNP) were investigated in the synthesis of 2'-deoxy, 2',3'-dideoxy and arabinonucleoside derivatives. *Lr*NDT-IMER catalyzed the synthesis of 5-fluoro-2'-deoxyuridine and 5-iodo-2'-deoxyuridine in 65-59% conversion yield, while *Cp*UP/*Ah*PNP-IMER provided the best results for the preparation of arabinosyladenine (60% conversion yield).

Both IMERs proved to be promising alternatives to chemical routes for the synthesis of nucleoside analogues. The developed in-flow system represents a powerful tool for the fast production on analytical scale of nucleosides for preliminary biological tests.

Keywords: Biocatalysis; Immobilized enzyme reactors; Nucleoside analogues; Nucleoside 2'-deoxyribosyltransferases; Nucleoside phosphorylases.

1. Introduction

The use of enzymes for the synthesis of active pharmaceutical ingredients (APIs) represents an interesting alternative to classical chemical routes. In particular, the intrinsic selectivity of enzymes allows the reduction of synthetic steps and their natural ability to work under mild conditions makes the production bioprocess eco-friendly (Busacca et al., 2011; Pollard and Woodley, 2007; Truppo, 2017; Woodley, 2008).

In addition, the immobilization of the enzyme to a solid support allows its stabilization and, consequently, the exploitation of more severe conditions (pH, temperature, solvents), as well as the reuse of the biocatalyst for multiple cycles (Bernal et al., 2018; Sheldon and Woodley, 2018).

Immobilized enzymes can be used in batch or in a system operating under continuous flow conditions (in-flow). In-flow reactions offer advantages compared to those performed in batch, resulting in an increase in productivity. Moreover, flow reactors can be prepared on different scales (from analytical to production scale). For the development of bioreactors on analytical scale, enzyme immobilization is generally performed directly in the column, thus creating an immobilized enzyme reactor (IMER) (Fang et al., 2012). IMERs can be used for rapid preparation of small quantities (microscale) of new products as required for drug discovery (Britton et al., 2018; Fang et al., 2012; Girelli and Mattei, 2005; Tamborini et al., 2018).

The choice of the immobilization carrier is an important issue to preserve enzymatic activity. In this context, monoliths emerged as interesting carriers for the preparation of IMERs due to their attractive features: high permeability and low back-pressure; accessibility of the immobilized macromolecule thanks to the high porosity; possibility

to use different immobilization methods and chemistries; stability (Calleri et al., 2012; Vlakh and Tennikova, 2013a, 2013b). For these reasons, monolithic supports represent an advancement compared to more common beads and particle columns.

In recent years, in-flow IMERs have been applied to the synthesis of different classes of APIs or pharmaceutical intermediates (Britton et al., 2018; Naldi et al., 2018; Tamborini et al., 2018). Among APIs, nucleoside analogues represent intriguing targets since their biocatalyzed synthesis can overcome different drawbacks of the classical chemical process used for their production (Ding et al., 2010; Fresco-Taboada et al., 2013; Mikhailopulo, 2007). Nucleoside phosphorylases (NPs) have proven their potential for the synthesis of modified nucleosides, which find applications as antiviral and antitumor agents. NPs can act on ribo- or 2'-deoxyribosylnucleosides by a reversible phosphorolytic reaction, resulting in the formation of the corresponding nucleobase and glycosyl moiety. The NP-catalyzed transfer of the sugar residue to a second nucleobase leads to the production of a new nucleoside (transglycosylation reaction) (Cattaneo et al., 2017; Fresco-Taboada et al., 2013). NPs are classified into families that possess different substrate specificity (for purine or pyrimidine nucleosides). Pyrimidine nucleoside phosphorylases have been successfully employed in the pyrimidine-pyrimidine transglycosylation reaction (Serra et al., 2013a, 2011), while the coupling of two NPs is often necessary to obtain the desired nucleoside when the transglycosylation occurs between a purine and a pyrimidine base. Recently, using in-flow bioreactors, purine-purine transglycosylation catalyzed by a purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNP) has been investigated (Calleri et al., 2015). Moreover, the in-flow bi-enzymatic tranglycosylation has also been described by connecting in

series two bioreactors containing a uridine phosphorylase from *Clostridium perfrigens* (*Cp*UP) and *Ah*PNP (Cattaneo et al., 2017) .

A different class of enzymes, 2'-deoxyribosyltransferases (NDTs), has also been used in batch for the synthesis of modified nucleosides (Fernández-Lucas et al., 2013, 2012, 2011, 2010). Reactions catalyzed by NDTs consist in the exchange between a nucleobase of a 2'-deoxyribonucleoside and a free nucleobase in one-step, with regio- and stereoselectivity (Fernández-Lucas et al., 2010; Fresco-Taboada et al., 2013; Huang et al., 1983). Two classes of NDTs can be distinguished: NDT type I (PDT), which catalyzes the deoxyribose group transfer exclusively between purines, and NDT type II (NDT), which catalyzes the transfer between purines and/or pyrimidines (Becker and Brendel, 1996; Holguin and Cardinaud, 1975; Kaminski, 2002).

The mechanism of NDTs shows to be similar to retaining glycoside hydrolases. Glycosyltransfer reaction occurs via double-displacement mechanism involving the formation of a covalently bound glycosyl-enzyme intermediate. In addition, NDTs perform acid/base catalysis similarly to glycosidases (Danzin and Cardinaud, 1974; Del Arco et al., 2019; Fresco-Taboada et al., 2018, 2013; Huang et al., 1983; Porter et al., 1995; Short et al., 1996).

Among the described NDTs, nucleoside 2'-deoxyribosyltransferase from *Lactobacillus reuteri* (*Lr*NDT) has been revealed as suitable biocatalyst for the synthesis of nucleoside analogues with therapeutic activity, showing the highest specific activity as well as catalytic efficiency (Fernández-Lucas et al., 2011, 2010). NDTs proceed through a ping-pong bi-bi mechanism. In the first step, the catalytic Glu residue attacks the anomeric C1' releasing the nucleobase, which leads to the glycosylated intermediate. In a second step, the second nucleobase attacks the glycosylated intermediate (transglycosylation)

to generate the corresponding nucleoside. Interestingly, a hydrolase function was also described in absence of nucleobase acceptors (Smar et al., 1991) or at long reaction times (Huang et al., 1983).

In this work, the preparation of an *Lr*NDT-IMER on a monolithic epoxy silica column is described for the first time. The obtained bioreactor has been applied to the synthesis of selected nucleosides of pharmaceutical interest. In addition, the co-immobilization of *Cp*UP and *Ah*PNP has also been investigated using a monolithic aminopropyl silica carrier. The two different bioreactors were included in a chromatographic system for in-flow reactions and tested in the preparation of different nucleosides on analytical scale in order to find the most adequate system for the synthesis of analogues modified at the base and/or at the sugar moiety.

2. Materials and methods

2.1. Chemicals and reagents

Inosine (Ino), 2',3'-dideoxyuridine (ddUrd), 5-iodouracil (IUra), 5-iodo-2'-deoxyuridine (IdUrd), 2-(N-Morpholino)ethanesulfonic acid (MES), methanol, glutaraldehyde, sodium cyanoborohydride, glycine, Bradford reagent and formic acid were purchased from Sigma-Aldrich (Milan, Italy). Hypoxanthine (Hpx), adenine (Ade), 2'-deoxyadenosine (dAdo), uracil (Ura), 2',3'-dideoxyinosine (ddIno), 5-fluorouracil (FUra) and 5-fluoro-2'-deoxyuridine (FdUrd) were from Alfa Aesar (Novachimica, Cinisello Balsamo, Italy). 2'-Deoxyuridine (dUrd) was supplied by Pro.Bio.Sint s.r.l. (Varese, Italy). Arabinosyluracil (araU) and arabinosyladenine (araA) were purchased from Jena Bioscience (Jena, Germany). Potassium dihydrogen phosphate and monoethanolamine were from Carlo Erba (Cornaredo, Italy). Ammonium sulphate was purchased from Merck KGaA

(Darmstadt, Germany). Deionized water was obtained from a Milli-Q® Integral purification system from Merck KGaA (Darmstadt, Germany).

Chromolith® Flash aminopropyl silica and WP300 Epoxy silica monolithic columns (4.6x25 mm) were kindly provided by Merck KGaA (Darmstadt, Germany). Analytical Symmetry Shield RP18 (4.6x250 mm, 5 µm, 100Å) and semi-preparative Atlantis® T3 Prep C18 (10x150 mm, 5 µm, 100 Å) columns were from Waters (Milford, MA, USA).

Uridine phosphorylase from *Clostridium perfringens* (*Cp*UP) and purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNP) were supplied by Gnosis S.p.A. (Desio, Italy) (Cattaneo et al., 2017).

2.2. Production of nucleoside 2'-deoxyribosyltransferase from Lactobacillus reuteri (LrNDT)

The *ndt* gene encoding *Lr*NDT was cloned into the NdeI-BamHI site of the expression vector pET28a(+) (Novagen, USA) generating the recombinant plasmid pET28a (+)-*Lr*NDT_{HisTh}, which provided a N-terminal His-Tag fusion *Lr*NDT containing a thrombin cleavage sequence between the tag and the enzyme (*Lr*NDT_{HisTh}). Recombinant enzyme was produced by *E. Coli* BL21 (DE3) cells harbouring pET28a (+)-*Lr*NDT_{HisTh} grown at 37 °C on LB (Luria Bertani) medium with kanamycin (50 µg/mL). Overexpression of *Lr*NDT_{HisTh} was induced with 0.5 mM IPTG for 2.5 h at the same temperature. Cells were then harvested by centrifugation at 3500 × *g* for 10 min, suspended in buffer A (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole buffer, pH 7.5) and disrupted by sonication on ice employing a Digital Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT, USA). The resulting cell extract was applied onto a 5 mL BioScale ™ Mini Profinity™ IMAC Cartridge (Bio-Rad, USA) equilibrated with buffer A and washed at a flow rate of 1 mL/min until the eluate contained no protein. Adsorbed protein was then

eluted with a linear gradient of 10 to 500 mM imidazole in 20 mM sodium phosphate buffer, 500 mM NaCl, pH 7.5. The presence of *Lr*NDT$_{HisTh}$ was detected by western-blot using monoclonal anti-polyHistidine Peroxidase Conjugate antibody (Sigma-Aldrich, USA). The protein fractions containing pure *Lr*NDT$_{HisTh}$ enzyme were pooled. Finally, His-tag was removed by the Thrombin Clean Cleavage™ kit (Sigma Aldrich, USA). The resulting pure enzyme was analysed by SDS-PAGE using a gel containing 15% acrylamide, as shown in **Fig. 1** (Laemmli, 1970).

2.3. In-solution activity assays

The activity of *Cp*UP, *Ah*PNP and *Lr*NDT was evaluated before enzyme immobilization following protocols from literature (Fernández-Lucas et al., 2010; Serra et al., 2013b; Ubiali et al., 2012).

In particular, 25 µL of 1:10 diluted *Cp*UP (7 mg/mL) or *Ah*PNP (21.5 mg/mL) solution were added to a 5 mM substrate solution (dUrd for *Cp*UP, Ino for *Ah*PNP) in 50 mM phosphate buffer, pH 7.5 (10 mL). Reactions were performed at room temperature under continuous stirring and monitored after 2, 4, 6 and 8 min. Reactions were stopped by removal of the enzyme through ultrafiltration (10 kDa MWCO centrifugal filter devices, 13200 rpm, 4 °C, 2 min) and samples were analyzed by RPLC-UV after 1:10 dilution (Serra et al., 2013b; Ubiali et al., 2012).

With regard to *Lr*NDT, 0.34 µg of enzyme were incubated with 10 mM dUrd and 10 mM Ade in 50 mM MES, pH 6.5 (40 µL) at 40 °C under continuous stirring. The enzyme was inactivated after 5 min by adding 40 µL of cold methanol to the mixture and heating at 95 °C for 5 min. Samples were centrifuged for 2 min at 9000 rcf (9205 rpm) and 50 µL of the supernatant were 1:25 diluted in the mobile phase before RPLC-UV analysis (Fernández-Lucas et al., 2010).

One international unit (IU) of enzyme activity corresponds to the amount of enzyme required to convert one μmol of substrate into product per minute. Enzyme activity was calculated as:

$$\frac{\text{substrate concentration (mM)} \times \text{conversion (\%)}}{\text{time (min)}} \times \text{vol (mL)} \Big/ \text{mg of enzyme}$$

and was expressed in IU/mg. Percentages of conversion were calculated from RPLC-UV analyses of the reaction mixtures as:

$$\frac{\text{product area}}{\text{product area} + \text{substrate area}} \times 100$$

2.4. Enzyme immobilization

The co-immobilization of *Cp*UP and *Ah*PNP was performed as already described in a previous work (Cattaneo et al., 2017), but on a Chromolith® Flash aminopropyl silica monolithic support (4.6x25 mm) instead of a particle column. The support was equilibrated with 50 mM phosphate buffer, pH 7.5 (20 min, 0.5 mL/min) and activated by pumping a 10 vol% glutaraldehyde solution in the same buffer (5 h, 0.5 mL/min). After washing with 100 mM phosphate buffer, pH 7.5 (10 min, 0.5 mL/min), 10 mL of a 2 mg/mL enzymatic solution in the same buffer (containing 10 mg of *Cp*UP and 10 mg of *Ah*PNP) were recirculated through the column overnight at 0.2 mL/min. The reduction of imines was then performed at 0.2 mL/min with 100 mM sodium cyanoborohydride solution (in 100 mM phosphate buffer, pH 7.5), for 1.5 h. After washing the support with 100 mM phosphate buffer, pH 7.5 (25 min, 0.2 mL/min), a 100 mM monoethanolamine solution in the same buffer was pumped at 0.2 mL/min for 2 h for the blocking of the unreacted functional groups. The support was washed for 25 min with 100 mM phosphate buffer, pH 7.5.

*Lr*NDT was immobilized on a Chromolith® Flash WP300 Epoxy silica monolithic column (4.6x25 mm) by an *in situ* procedure previously developed (Temporini et al., 2006; Tengattini et al., 2018). The support was equilibrated with 50 mM phosphate buffer and 1.9 M ammonium sulphate, pH 8.0 (1 h, 0.5 mL/min) and 10 mL of enzymatic solution containing 3.5 mg of *Lr*NDT in the same buffer were recirculated in the column at 0.5 mL/min for 4 h, reversing the support every 15 min. The monolithic column was then washed overnight with 10 mM phosphate buffer, pH 6.0 at 0.05 mL/min. Blocking of the unreacted epoxide groups was performed by flowing a 1M glycine solution in 50 mM phosphate buffer, pH 7.0 (2h, 0.5 mL/min). The IMER was equilibrated with 50 mM MES buffer, pH 6.5.

For both bioreactors, the immobilization yield was estimated by the Bradford assay and IMERs were stored at 4 °C when not in use.

2.5. In-flow activity assays

The activity of immobilized *Cp*UP and *Ah*PNP was assessed by evaluating the on-column phosphorolysis of dUrd and Ino, respectively (Cattaneo et al., 2017). After discarding the solution eluting from the column corresponding to the dead volume of the system, 0.5 mL of a 20 mM dUrd (for *Cp*UP) or Ino (for *Ah*PNP) solution in 100 mM phosphate buffer, pH 7.5 were pumped through the *Cp*UP/*Ah*PNP-IMER (flow rate 0.5 mL/min, temperature 37 °C). Each reaction mixture was collected after a single passage through the IMER and 1:40 diluted in the mobile phase before the chromatographic analysis. Percentages of conversion were calculated as:

$$\frac{\text{product area}}{\text{product area} + \text{substrate area}} \times 100$$

After enzyme immobilization, *Lr*NDT activity was evaluated by adapting a previously described in-solution assay (Fernández-Lucas et al., 2010) to the in-flow system. A

solution of 5 mM dUrd and 5 mM Ade, in 50 mM MES buffer (pH 6.5) or in 10 mM phosphate buffer (pH 7.0), was pumped through the bioreactor at different flow rates (0.1, 0.2 and 0.5 mL/min), at 37 °C. The solution eluting from the column corresponding to the dead volume of the system was sent to the waste and 0.5 mL of reaction mixture were collected after a single passage through the column at each flow rate. Samples were analyzed off-line by HPLC-UV after 1:25 dilution in the mobile phase. The percentage of conversion was calculated as:

$$\frac{\text{dAdo area}}{\text{dAdo area} + \text{Ade area}} \times 100$$

2.6. In-flow synthesis of nucleosides

Reactions were carried out by recirculating for 24 h in each IMER a 5 mL solution of 5 mM nucleobase and 10 mM sugar donor (for dAdo, ddIno, FdUrd and IdUrd) in 10 mM phosphate buffer, pH 7.0 at a temperature of 37 °C and using a flow rate of 0.5 mL/min. FdUrd was also synthesized starting from 20 mM sugar donor, while the production of IdUrd was carried out both at 25 and 37 °C. For araA, due to its low solubility (around 2 mM) (Cattaneo et al., 2017), 5 mL of 1 mM nucleobase + 2 mM sugar donor in 2 mM phosphate buffer, pH 7.0, were recirculated into each IMER; a temperature of 37 °C and a flow rate of 0.5 mL/min were applied.

Fractions (50 µL) of each reaction mixture were collected at fixed times (0, 0.5, 1, 2, 4, 6, 22 and 24 h) and 1:25 diluted in the mobile phase before RPLC-UV analysis for reaction monitoring. Since araA was produced starting from a lower substrate concentration, in this case the reaction mixture was diluted 1:10 before analysis.

Conversion yield was calculated at each time as:

$$\frac{\text{nucleoside product concentration (mM)}}{\text{nucleobase substrate concentration at } t_0 \text{(mM)}} \times 100$$

Product and substrate concentrations were calculated from calibration curves built in the concentration range between 0.05 and 1 mM.

2.7. Stability studies

The stability of dAdo, dUrd, FdUrd and IdUrd was investigated to assess enzymatic hydrolysis and degradation.

In-solution stability studies were performed by incubating 2 mL of a 5 (FdUrd and IdUrd) or 10 (dAdo and dUrd) mM solution of each nucleoside in 10 mM phosphate buffer, pH 7.0 at room temperature or 37 °C for 24 h. Stability in presence of the enzyme was evaluated after incubation of 17 μg of *Lr*NDT with a 5 (IdUrd) or 10 (dAdo and dUrd) mM nucleoside solution in 10 mM phosphate buffer, pH 7.0 (2 mL), at room temperature or 37 °C for 24 h. Solutions were 1:25 diluted in the mobile phase before RPLC-UV analysis. In-flow stability studies were carried out by recirculating a 5 (IdUrd) or 10 (dAdo and dUrd) mM nucleoside solution into *Lr*NDT-IMER for 24 h, under the same conditions employed for the in-flow reactions (medium: 5 mL of 10 mM phosphate buffer, pH 7.0; temperature: 37 °C; flow rate: 0.5 mL/min). Fractions (50 μL) of each reaction mixture were collected at fixed times (0, 0.5, 1, 2, 4, 6, 22 and 24 h) and 1:25 diluted in the mobile phase before LC-UV monitoring.

2.8. Chromatographic methods

Chromatographic analyses were performed by an Agilent HPLC series 1100 system (Santa Clara, CA, USA), equipped with mobile phase online degasser, quaternary pump, column thermostated compartment and variable wavelength detector. A Symmetry Shield RP18 column (4.6x250 mm, 5 μm, 100Å) from Waters (Milford, MA, USA) was used.

Analytical methods to separate and quantify substrates and products were developed for each reaction, keeping constant flow rate (1 mL/min) and UV detector wavelength (260 nm). Isocratic elution was applied to all samples.

For in-solution and in-flow activity assays, phosphorolysis of dUrd (for *Cp*UP activity) and Ino (*Ah*PNP) were monitored using the following chromatographic parameters: injection volume, 20 µL; mobile phase, 95/5 water/methanol; room temperature; analysis time, 10 min (dUrd) or 12 min (Ino). Instead, the transglycosylation conversion of dUrd and Ade into dAdo and Ura was analyzed to assess *Lr*NDT activity using the following method: injection volume, 10 µL; mobile phase, 95/5 10 mM phosphate buffer, pH 4.6/methanol; room temperature; analysis time, 35 min.

The chromatographic parameters employed to monitor the in-flow synthesis of dAdo and araA were: injection volume, 10 µL; mobile phase, 95/5 10 mM phosphate buffer, pH 4.6/methanol; room temperature; analysis time, 35 min (dAdo) or 30 min (araA). For the synthesis of ddIno: injection volume, 10 µL; mobile phase, 90/10 water/methanol; temperature, 35 °C; analysis time, 18 min. Synthesis of FdUrd and IdUrd: injection volume, 10 µL; mobile phase, 90/10 10 mM phosphate buffer, pH 4.6/methanol; temperature, 35 °C; analysis time, 18 min (FdUrd) or 20 min (IdUrd).

For stability studies, the same chromatographic methods applied to monitor the in-flow synthesis of nucleosides were employed.

The identity of each product was confirmed by LC-UV analysis of the corresponding commercial standard and by LC-MS/MS analysis of the reaction mixtures after 24 h of recirculation in each IMER. For LC-MS/MS analyses, the following chromatographic parameters were used: injection volume, 100 µL of non-diluted reaction mixture; mobile phase, 95/5 water/methanol (dAdo and araA) or 90/10 water+0.1% formic

acid/methanol (ddIno, FdUrd and IdUrd); flow rate, 1 mL/min; room temperature (dAdo and araA) or 35 °C (ddIno, FdUrd and IdUrd). The flow rate was split at the column outlet so that 0.15 mL/min reached the MS instrument. MS detection was performed by a LTQ linear ion trap mass spectrometer with an electrospray ionization (ESI) ion source (Thermo Finnigan, San Jose, CA, USA) using the instrumental conditions previously reported (Cattaneo et al., 2017).

Calibration curves were built for each nucleoside-nucleobase pair (dAdo-Ade, araA-Ade, ddIno-Hpx, FdUrd-FUra, IdUrd-IUra) in the concentration range 0.05-1 mM. Linear responses were obtained, giving the following results: $y = 8653.9x - 11.775$, $R^2 = 1$ for dAdo; $y = 9215.3x - 26.8$, $R^2 = 1$ for Ade; $y = 10499x + 55.572$, $R^2 = 0.9998$ for araA; $y = 5739.4x - 6.2605$, $R^2 = 0.9998$ for ddIno; $y = 6351.1x + 24.552$, $R^2 = 0.9998$ for Hpx; $y = 4809.4x + 11.136$, $R^2 = 0.9999$ for FdUrd; $y = 4374.2x + 28.753$, $R^2 = 1$ for FUra; $y = 1988.2x + 3.117$, $R^2 = 1$ for IdUrd; $y = 2278.2x + 7.5594$, $R^2 = 1$ for IUra.

2.9. Product purification

FdUrd was obtained by the recirculation of a 5 mL solution of 5 mM FUra and 20 mM dUrd in 10 mM phosphate buffer, pH 7.0 in *Lr*NDT-IMER for 1 h, at a temperature of 37 °C and using a flow rate of 0.5 mL/min. IdUrd was produced by recirculating a 5 mL solution of 5 mM IUra and 10 mM dUrd in 10 mM phosphate buffer, pH 7.0 in *Lr*NDT-IMER for 0.5 h, at 37 °C and 0.5 mL/min.

The resulting reaction mixture was divided into 5 aliquots (each one with a volume of 1 mL), which were injected in a semi-preparative column for product purification. An Atlantis® T3 Prep C18 column (10x150 mm, 5 µm, 100 Å) from Waters (Milford, MA, USA) was used. The following chromatographic parameters were applied: injection volume, 1 mL; mobile phase, 97/3 (FdUrd) or 90/10 (IdUrd) water/methanol; isocratic

elution; flow rate, 3 mL/min (FdUrd) or 4 mL/min (IdUrd); temperature, 35 °C; UV detector wavelength, 260 nm; analysis time, 25 min (FdUrd) or 22 min (IdUrd). For each analysis, the fraction eluting approximately between 18.5 and 22.5 min (corresponding to FdUrd peak) or between 16 and 20 min (corresponding to IdUrd peak) was collected. Water was removed from the collected fractions by a Laborota 4000 evaporator from Heidolph (Schwabach, Germany) and samples were subsequently lyophilized by a Modulyo freeze dryer from Edwards (Cinisello Balsamo, Italy).

Purified FdUrd and IdUrd were analyzed by RPLC-UV (for purity evaluation) using the same method employed to monitor their in-flow synthesis and characterized by NMR (recorded at 400 MHz with Bruker AVANCE DRX 400 spectrometer) by comparison with an authentic commercial standard.

3. Results and discussion

3.1. Preparation and characterization of the IMERs

Enzyme activity was tested prior to the immobilization by performing in-solution assays. The phosphorolysis of 2'-deoxyuridine (dUrd) by *Cp*UP and inosine (Ino) by *Ah*PNP was monitored to calculate the specific activity of the two NPs (Cattaneo et al., 2017), which resulted 16 IU/mg for *Cp*UP and 34 IU/mg for *Ah*PNP. *Lr*NDT activity was evaluated in the transglycosylation conversion of dUrd and adenine (Ade) to 2'-deoxyadenosine (dAdo) and uracil (Ura) (Fernández-Lucas et al., 2010) and was found to be 32 IU/mg.

IMERs were thus prepared by immobilizing the enzymes on monolithic supports. Chromolith® silica-based monolithic columns have been reported to be attractive supports for enzyme immobilization. In fact, monolithic supports exhibit micrometer-size flow-through pores that form a macroporous network and nanometer size

mesopores on the skeleton that result in fast kinetics and high binding properties (Temporini et al., 2006; Tengattini et al., 2018).

The NP-IMER was obtained by the co-immobilization of *Cp*UP and *Ah*PNP on an aminopropyl silica monolithic column functionalized with glutaraldehyde. The same immobilization chemistry was already described in literature for these enzymes (Calleri et al., 2015; Cattaneo et al., 2017). In particular, Calleri *et al.* described the successful immobilization of *Ah*PNP on an aminopropyl silica particle column and the use of the resulting *Ah*PNP-IMER for the synthesis of purine ribonucleosides (Calleri et al., 2015). In the paper by Cattaneo *et al.*, *Cp*UP and *Ah*PNP were co-immobilized on an aminopropyl silica particle-based support, which offered a high backpressure resulting in a loss of enzyme activity; the use of a *Cp*UP monolithic IMER and an *Ah*PNP particle bioreactor connected in series allowed to reduce the backpressure and to obtain the desired nucleosides (Cattaneo et al., 2017). To our knowledge, the present paper reports for the first time the development of a monolithic *Cp*UP/*Ah*PNP-IMER.

Similarly, for NDT-IMER, an epoxy monolithic support was selected for the immobilization. The choice was based on a previously reported study describing *Lr*NDT immobilization on epoxy-activated Sepabeads in batch (Fernández-Lucas et al., 2011). The present work also describes for the first time the immobilization of *Lr*NDT on a monolithic column.

For both bioreactors the immobilization was performed following *in situ* protocols (Cattaneo et al., 2017; Temporini et al., 2006; Tengattini et al., 2018). Immobilization yields were calculated by the Bradford assay, resulting in 34% for *Cp*UP/*Ah*PNP and 30% for *Lr*NDT (corresponding to 6.8 mg and 1 mg immobilized enzymes, respectively).

For *Cp*UP/*Ah*PNP-IMER, the activity of each immobilized enzyme was assessed separately by on-column phosphorolysis of dUrd for *Cp*UP and Ino for *Ah*PNP (Cattaneo et al., 2017). Reaction mixtures were analyzed off-line by HPLC, yielding a conversion of 35% for *Cp*UP (from dUrd to Ura) and 30% for *Ah*PNP (from Ino to Hpx).

To assess *Lr*NDT-IMER activity, the production of dAdo starting from dUrd and Ade was investigated by on-column tranglycosylation. For *Lr*NDT-IMER, the effect of the flow rate was also studied by performing the assay at 0.1, 0.2 and 0.5 mL/min. In addition, two reaction buffers were investigated: 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.5, usually employed for standard NDT activity assays (Fernández-Lucas et al., 2010); 10 mM phosphate buffer, pH 7.0 in view of the comparison with the *Cp*UP/*Ah*PNP biocatalytic route, since the reaction catalyzed by NPs requires the presence of inorganic phosphate. Regardless of the flow rate and the buffer used, the same conversion yield (approximately 80%) was reached. Conversion yields were calculated as reported in Materials and methods.

The reuse and stability of enzymes are prerequisites for their application in IMERs. Therefore, the same assays were used to evaluate the stability of immobilized enzymes over time. To date, *Cp*UP/*Ah*PNP-IMER retained its catalytic activity over 12 reactions in 18 months, *Lr*NDT-IMER over 22 reactions in 16 months.

3.2. Assessment of the in-flow performances of the IMERs by a reference reaction

After the confirmation that the enzymes maintained their activity upon immobilization, *Cp*UP/*Ah*PNP-IMER and *Lr*NDT-IMER were tested by performing the same transglycosylation reaction for the synthesis of dAdo.

IMERs were placed in a chromatographic system, and a 5 mL reaction mixture containing sugar donor (10 mM dUrd) and nucleobase (5 mM Ade) was continuously pumped

through each bioreactor at a constant flow rate (0.5 mL/min) and temperature (37 °C) to achieve the biotransformation (Cattaneo et al., 2017). In order to have the ideal environment for both IMERs, 10 mM phosphate buffer pH 7.0 was chosen for the reactions. In fact, *Lr*NDT was proven to express the best activity between pH 5.0 and 7.0 (Fernández-Lucas et al., 2010), while for *Cp*UP- and *Ah*PNP-IMERs the activity was not influenced in the pH range between 7.0 and 7.5 (Cattaneo et al., 2017). The reaction progress was monitored by HPLC.

In the synthesis of dAdo, both bioreactors allowed to reach high conversion yields in a short time, further proving the good performance of the enzymes. The reaction performed in *Lr*NDT-IMER was slightly more efficient and fast (maximum conversion yield = 88% in 1 h) compared to the one catalyzed by the bi-enzymatic bioreactor (maximum conversion yield = 84 % in 2 h), as shown in **Fig. 2**. After maximum conversion, a progressive decrease in the product concentration was observed (**Fig. 2**) and the chromatographic analysis revealed the presence of secondary reaction products. Therefore, the drop in the conversion yield was ascribed to side reactions.

To confirm this hypothesis, stability studies on dAdo were performed. At first, stability was assayed in batch. Two solutions of dAdo were incubated for 24 h at room temperature and 37 °C. The nucleoside proved to be stable at both temperatures, suggesting that its degradation over time was not spontaneous. Nucleoside stability in solution was also investigated in presence of *Lr*NDT because the formation of secondary products was particularly marked using this enzyme. After a 24 h-incubation, no secondary products were formed. Only a partial hydrolysis of dAdo in Ade was observed at room temperature (1% conversion) and, to a greater extent, at 37 °C (3% conversion).

The in-flow behavior of dAdo was also examined. A 10 mM dAdo solution was recirculated into *Lr*NDT-IMER in the same conditions employed for the transglycosylation reaction. Chromatographic analyses of the solution at fixed times revealed a partial hydrolysis of dAdo in Ade and the formation of secondary products with the same retention times observed in the synthetic reaction. Results showed that the formation of secondary products was promoted by the interaction of the IMER with dAdo.

3.3. In-flow microscale synthesis of pharmaceutically relevant nucleoside analogues

The two bioreactors were tested in the microscale production of nucleoside analogues of pharmaceutical interest as antiviral and antitumor agents. First, the synthesis of two antiviral purinic nucleosides was investigated: arabinosyladenine (araA) and 2',3'-dideoxyinosine (ddIno). Due to the poor solubility of araA (around 2 mM) (Cattaneo et al., 2017), the reaction was performed using a low concentration of reagents (1 mM Ade nucleobase + 2 mM araU sugar donor in 2 mM phosphate buffer, pH 7.0), while for ddIno higher concentrations were used (5 mM Hpx nucleobase + 10 mM ddUrd sugar donor in 10 mM phosphate buffer, pH 7.0). The maximum conversion yields are reported in **Table 1**.

*Lr*NDT was the first NDT found to exhibit an arabinosyltransferase activity (Fernández-Lucas et al., 2010). However, it hardly catalyzed the in-flow synthesis of araA, reaching a conversion <2% in 24 h (**Table 1**). Even if the possibility to obtain arabinonucleosides by a NDT mono-enzymatic reactor would be intriguing, the use of NPs turned out to be more convenient. In fact, the reaction performed in the bi-enzymatic IMER resulted in a conversion yield of 60% in 24 h (**Table 1**).

On the contrary, for ddIno the reaction was found to be more efficient using *Lr*NDT, with a conversion yield of 23% in 22h (**Table 1**). However, two secondary products grew over time in parallel to the product, suggesting a connection between these compounds. LC-MS/MS analyses supported this theory and suggested that the two secondary products are composed of isomers of ddIno and/or its substrate (Hpx), as shown by the extracted ion chromatogram of their *m/z*. The conversion catalyzed by *Cp*UP and *Ah*PNP was less efficient (conversion yield = 12% in 24 h, **Table 1**) but more specific, since only substrates and products were detected in the chromatographic reaction monitoring.

The synthesis in batch of araA and ddIno by a one-pot, bienzymatic transglycosylation was previously described by using immobilized *Cp*UP and *Ah*PNP achieving 74 and 44% conversion after 48 and 24 hours, respectively (Serra et al., 2013b).

The synthesis of two pyrimidinic nucleosides was then investigated: 5-fluoro-2'-deoxyuridine (FdUrd), an antineoplastic agent, and 5-iodo-2'-deoxyuridine (IdUrd), an antiherpes drug.

Previously, pyrimidine nucleoside phosphorylase from *Bacillus subtilis* (*Bs*PyNP) and thymidine phosphorylase from *Escherichia coli* (*Ec*TP) were immobilized and used for the batch synthesis of FdUrd and IdUrd by transglycosylation in fully aqueous medium. The synthesis of these compounds using dUrd as sugar donor proceeded at a similar conversion (68, 57% for *Bs*PyNP and 62, 56% for *Ec*TP, respectively, after about 10 hours) (Serra et al., 2013a).

In this work, the synthesis of FdUrd and IdUrd using dAdo as sugar donor gave comparable results in the two bioreactors. For FdUrd, a conversion yield of approximately 40% was achieved in 1 h of recirculation. IdUrd production showed a faster kinetics, but a lower conversion yield (approximately 25% in 0.5 h, **Table 1**). The

chromatographic analysis of reaction mixtures revealed the progressive formation of secondary products. The same species obtained in the in-flow stability study of dAdo were observed also in the synthetic reactions (data not shown). In addition, it was possible to highlight the coelution of one of these species with FdUrd; therefore, the conversion yield for this reaction was calculated on FUra consumption instead of FdUrd production.

The use of *Lr*NDT-based bioreactor to catalyze the synthesis of FdUrd and IdUrd was found more convenient compared to *Cp*UP/*Ah*PNP-IMER. In fact, similar conversion percentages were obtained, despite the lower amount of immobilized *Lr*NDT enzyme (1 mg *vs* 6.8 mg). Noteworthy, NDTs allow the synthesis of nucleoside analogues using a single catalyst.

Therefore, the mono-enzymatic bioreactor was selected for further studies in order to increase conversion yields. In particular, the sugar donor was changed in order to avoid undesired degradation or enzymatic secondary reactions as observed when dAdo was used. Thus, dUrd was employed for the synthesis of FdUrd and IdUrd since it was described as the best sugar donor for *Lr*NDT (Fernández-Lucas et al., 2010). The conversion for FdUrd improved from 37% in 2 h to 50% in 0.5 h (**Table 1**). For IdUrd, the maximum conversion yield increased from 26% using dAdo to 59% with dUrd (**Table 1**). No side reactions were observed during FdUrd and IdUrd synthesis starting from dUrd. In fact, only peaks corresponding to the expected substrates and products were detected in the chromatographic reaction monitoring, further confirming the correlation of the previously observed secondary products with dAdo degradation. However, a partial hydrolysis of the products was noticed after reaching the maximum conversion (**Fig. 3**).

Stability studies on dUrd, FdUrd and IdUrd were performed and these products proved to be stable in solution, both at room temperature and at 37 °C, suggesting that their hydrolysis was not spontaneous. The stability of the sugar donor (dUrd) and a representative product (IdUrd) was also assessed in the presence of the enzyme. Therefore, each compound was individually incubated with *Lr*NDT; the enzyme catalyzed the hydrolysis of both nucleosides at room temperature and, to a greater extent, at 37 °C. In-flow experiments were also carried out in *Lr*NDT-IMER and resulted in a nearly complete hydrolysis of dUrd or IdUrd (97-100% conversion in 24 h). These data suggest that *Lr*NDT catalyzes the hydrolysis of nucleosides. Probably, the presence of two acid residues in the active site allows the NDT to catalyze the hydrolysis of the glyosidic bond in the nucleoside product, similarly to the mechanism used by glycosidases (Danzin and Cardinaud, 1974; Del Arco et al., 2019; Short et al., 1996). This effect can be responsible for the reduction of the yields obtained in the synthetic process.

According to the results, it seems that *Lr*NDT is able to catalyze the hydrolysis of the glycosidic bond as described for NDT from *Lactobacillus leichmannii* (Huang et al., 1983). These results are also reinforced by the *in silico* approach reported by Del Arco *et al.* (Del Arco et al., 2019), which shows the evidence of two independent reactions, called nucleobase release (first reaction) and transglycosylation (second reaction). As shown by Smar and coworkers (Smar et al., 1991), in the absence of a nucleobase acceptor the reaction proceeds towards the hydrolysis of the glycosyl intermediate. Moreover, it is reported that adenine is a competitive inhibitor of the first substrate for NDT from *Lactobacillus helveticus* (Cardinaud, 1978). In this sense, several factors, such as the absence of nucleobase acceptor (Smar et al., 1991), long reaction times (Huang et al.,

1983) or inhibition by substrate or product (Cardinaud, 1978) promote the competition of hydrolisis with the desired synthetic process. This could strongly affect the yields reducing the concentration of the tranglycosylation product, and the process seems to be under kinetic control.

Conversion yields in the transglycosylation reaction can be improved by increasing the sugar donor concentration (which agrees with a competitive inhibition by adenine), as observed for FdUrd. For this nucleoside, a conversion yield of 65% (corresponding to around 4 mg of FdUrd) was reached after 1 h when a 20 mM dUrd concentration was used (**Fig. 3**, **panel A**).

Instead, while the degree of nucleoside enzymatic hydrolysis was reduced in solution at room temperature, the synthesis of IdUrd in *Lr*NDT-IMER at 25 °C was not beneficial. Temperature reduction enhanced product stability over time (a lower degree of hydrolysis was observed in the monitored 24 h), but the kinetics of the synthesis was slower; globally, the conversion yield decreased (**Fig. 3**, **panel B**) compared to the reaction at 37 °C. In particular, at 25 °C the highest conversion yield was 49% (after 6 h), while at 37 °C 59% (corresponding to about 5 mg of IdUrd) was obtained in 0.5 h.

3.4. Purification of products FdUrd and IdUrd

A method for purification was developed on a semi-preparative scale for FdUrd and IdUrd. FdUrd was produced in *Lr*NDT-IMER starting from 20 mM dUrd. The recirculation was stopped after 1 h and the resulting mixture was loaded on the semi-preparative column. Fractions including FdUrd eluting from the column were collected and freeze-dried. The identity and purity of the nucleoside analogue were then verified by RPLC-UV (purity >99%) and NMR analyses comparing the purified FdUrd with its commercial reference standard.

For IdUrd, the in-flow reaction was performed in 0.5 h using 10 mM of sugar donor (dUrd). After this time, the resulting mixture was loaded on the semi-preparative column to isolate the target nucleoside. RPLC-UV (purity >99%) and NMR analyses confirmed IdUrd identity and purity.

4. Conclusions

The enzymatic microscale synthesis of different nucleoside analogues was performed using a bi-enzymatic (obtained by co-immobilizing two NPs) and a mono-enzymatic (immobilization of one NDT) bioreactors. The combination of *Cp*UP and *Ah*PNP provided the best results for the synthesis of araA, according to previous data obtained using two mono-enzymatic IMERs.

Instead, *Lr*NDT-IMER gave promising results for 2'-deoxyribonucleosides, which were synthesized with 50-88% conversion yields using dUrd as sugar donor; the highest conversions achieved for FdUrd and IdUrd were comparable to those obtained in batch using *Bs*PyNP and *Ec*TP, but with reduced reaction times (0.5-1 h in-flow compared to 10 h in batch). Both bioreactors are promising for the rapid preparation of small amounts of nucleoside analogues as required for drug discovery.

E-supplementary data for this work can be found in e-version of this paper online.

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Figure captions

Fig. 1. SDS-PAGE analysis of soluble *Lr*NDT$_{HisTh}$ (**A**): Precision Plus Protein™ prestained standard from BioRad used as a molecular weight marker (lane 1); supernatant obtained after centrifugation of the lysed cells (lane 2); purified *Lr*NDT$_{HisTh}$ after affinity chromatography (lane 3). Western Blot of soluble *Lr*NDT$_{HisTh}$ (**B**): Precision Plus Protein™ prestained standard from BioRad used as a molecular weight marker (lane 1); supernatant obtained after centrifugation of the lysed cells (lane 2); purified *Lr*NDT$_{HisTh}$ after affinity chromatography (lane 3).

Fig. 2. Monitoring over time of the synthesis of dAdo performed in *Lr*NDT-IMER (blue) or *Cp*UP/*Ah*PNP-IMER (red). Conversion yields were calculated as the ratio between molar product concentration at each time and molar substrate concentration at t = 0 h.

Fig. 3. Monitoring over time of the synthesis of FdUrd (**A**) and IdUrd (**B**) in *Lr*NDT-IMER using dAdo or dUrd as sugar donor. For FdUrd, data obtained from the reactions carried out starting from 10 mM and 20 mM dUrd are reported. For IdUrd, data obtained from the reactions carried out at 37 and 25 °C are shown. Conversion yields were calculated as the ratio between molar product concentration at each time and molar substrate concentration at t = 0 h.