The SNAP-tag technology revised: an effective chemo-enzymatic approach by using a universal azide-based substrate

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ABSTRACT
SNAP-tag is a powerful technology for the labelling of protein/enzymes by using benzyl-guanine (BG) derivatives as substrates. Although commercially available or ad hoc produced, their synthesis and purification are necessary, increasing time and costs. To address this limitation, here we suggest a revision of this methodology, by performing a chemo-enzymatic approach, by using a BG-substrate containing an azide group appropriately distanced by a spacer from the benzyl ring. The SNAP-tag and its relative thermostable version (SsOGT-H) proved to be very active on this substrate. The stability of these tags upon enzymatic reaction makes possible the exposition to the solvent of the azide-moiety linked to the catalytic cysteine, compatible for the subsequent conjugation with DBCO-derivatives by azide-alkyne Huisgen cycloaddition. Our studies propose a strengthening and an improvement in terms of biotechnological applications for this self-labelling protein-tag.

1. Introduction
The advent of the self-labelling protein-tags (SLPs) has led to a huge push in modern biotechnology, especially in the field of cell biology, where auto-fluorescent proteins (AFPs) for a long time dominated for their versatility in the localisation experiments of proteins, organelles, and membranes. But the use of SLPs clearly goes beyond: they catalyse the covalent, highly specific and irreversible attachment of a part of their synthetic ligands upon reaction. This offers the opportunity to label them by conjugation to those ligands of an infinite number of chemical groups, such as fluorescent dyes, affinity molecules, or solid surfaces, expanding the application fields. Among SLPs, of particular note are the HaloTag, the SpyTag, the SNAP- and the CLIP-tag. The Promega HaloTag is a halo-alkane dehalogenase with a genetically modified active site, which reacts irreversibly with primary alkyl-halides.

SNAP-tag from New England Biolabs (NEB) is the engineered variant of the natural suicide human O6-methylguanine DNA-methyltransferase protein (hMGMT). Alkylated DNA-alkyltransferases (AGTs, MGMTs or OGTs, E.C. 2.1.1.63) are ubiquitous and conserved proteins involved in the repair of the DNA alkylation damage, in particular, they remove alkyl adducts at the level of O6-position on guanine base. The peculiar single-step mechanism are called “suicide enzymes,” in which the alkylated base is directly repaired by the irreversible transfer of the alkylic group from the damaged guanine to the catalytic cysteine in the protein active site. The protein is permanently inactivated upon the trans-alkylation reaction and susceptible to in vivo degradation via the proteasome.

In 2003, the group of Kai Johnsson developed a new strategy to exploit the hMGMT suicidal reaction in biotechnology, adopting a directed-evolution approach to engineer a variant to be used as an innovative protein-tag, that is, the SNAP-tag. The rationale behind the SNAP-tag technology is the low substrate specificity of some AGT proteins, being able to efficiently recognise also the O6-benzyl-guanine (BG) nucleobase. Likely, the reaction of these enzymes with BG-derivatives could happen: upon the irreversible transfer to the catalytic cysteine, they indeed demonstrated the specific labelling of the hMGMT with molecules, as fluorophores, previously conjugated to the 4-position of the BG benzyl ring. Because of the small dimension of this protein, it was mutagenized to abolish any DNA binding activity and utilised as protein-tag for the indirect labelling of proteins of interest genetically fused to it (Figure 1). Later, the same group further engineered the SNAP-tag to obtain the CLIP-tag, which specifically reacts with O6-benzyl-cytosine derivatives, expanding that technology for in vivo and in vitro multi-protein labelling.

Apart from cell biology and fluorescence imaging, hundreds of papers are present in the literature showing many applications of SNAP-tag in several fields, among which RNA-editing, the...
Figure 1. Single-step reaction vs chemo-enzymatic approach. (A) The SNAP-tag® technology is based on BG-derivatives singularly synthesised and purified, and not excluding that the conjugated chemical group (green sphere) could affect the enzymatic reaction rate. (B) The SNAP-tag® technology revised uses a unique and universal azide BG-derivative, converting SNAP-tag® in a clickable form, prone to perform a fast and efficient cycloaddition with DBCO-based chemical groups. POI, protein of interest genetically fused to the SNAP-tag®.

Following the same approach, Perugini and co-workers expanded this technology to extremophilic organisms and to all the applications which require harsh reaction conditions, not fully suitable for the employing of the mesophilic SNAP-tag®. To this aim, they developed a “thermo-SNAP-tag®” by the production of a variant of the OGT from *Saccharolobus solfataricus* (previously *Sulfolobus solfataricus*, *Ss*OGT-H5, hereinafter H5), an enzyme which revealed extremely resistant to high temperature, high ionic strength, proteases attack, and, in general, to common physical and chemical denaturants. The intrinsic stability of H5 made it compatible with expression and utilisation in vivo as protein-tag in thermophilic organisms, as *Thermus thermophilus* and *Sulfolobus islandicus* as well as in an in vitro expression system using *Sulfolobus* lysates. Recently, H5 became a part of the new ASL system, which was particularly useful for the in vivo immobilisation and contemporary labelling of proteins and enzymes of interest, stabilising them without any purification procedures needed.

SNAP-tag® technology is essentially based on BG-substrates: although many of them are commercially available, the possibility of conjugation of infinite desired molecules to the 4-position on BG leads to the synthesis of ad hoc substrates. This is generally possible through the crosslinking reaction of the so-called “BG-building block” (such as the amine-reactive BG-NH2) with NHS-ester derivative compounds. The main disadvantage is the need to purify the final compounds before the reaction with the enzyme, increasing the times and costs of the experiments. Furthermore, the presence of chemical groups conjugated to the benzyl moiety of the BG could affect the reaction efficiency of the SNAP-tag®, sometimes making this enzyme not fully applicable to particular requests.

In this work, we analysed and confirmed the catalytic dependence of SNAP-tag® and H5 by several substrates having different chemical groups conjugated to the O6-position of the guanine. To overcome these limitations, in the current study we suggest a further improvement of this technology with the application of a chemo-enzymatic approach, by using a unique and universalazole decorated BG-derivative, converting SNAP-tag® in a clickable form, prone to perform a fast and efficient cycloaddition with DBCO-based chemical groups. Here, we successfully proved the labelling of the SNAP-tag® with several DBCO-based fluorophores and the covalent immobilisation of this protein on alkyne-coated surface sensors.

2. Materials and methods

2.1. Reagents

BG was from Activate Scientific GmbH (UK), whereas MGPA was a gift of Prof D. Prosperi (University of Bicocca, Milan, Italy). SNAP-Vista® Green (SVG), SNAP Cell® Block (SCB), SNAP Cell®-430 (SC430), BG-PEG-NH2 (BGPA), pSNAP-tag(m) plasmid, DNA restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs (USA). Molecular biology kits for plasmid preparations were from Macherey-Nagel GmbH (Germany). Oligonucleotides synthesis and DNA sequencing service were performed by Eurofins Genomics (Germany). BDP FL alklyne, BDP FL DBCO, Cy5 DBCO were purchased from Lumiprobe GmbH (Germany). DBCO-Peg-Fluor 545, Tris(2-carboxyethyl)phosphin (TCEP), Tris ((1-benzyl-1H-1,2,3-triazol-4-yl)-methyl)amine (TBA) from Sigma-Aldrich (St. Louis, MO). PierceTM Premium Grade Sulfo-NHS (N-hydroxy-sulfo-succinimide) and PierceTM Premium Grade 1-ethyl-3-(3-dimethyl-amino-propyl)-
2.2. Compounds synthesis: general procedures

1H (400 MHz) and 13C (100 MHz) NMR spectra were measured on Bruker Advance Neo 400 MHz spectrometer. Chemical shifts were referenced to the residual solvent signal (CDCl3: δH = 7.26, δC = 77.0; DMSO: δH = 2.50 δC = 39.5). Low-resolution ESI-MS were obtained on an LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. IR spectra were registered on Shimadzu DR 8001 spectrophotometer. Silica gel 60 (70–230 mesh) used for gravity column chromatography (CC) was purchased from Macherey-Nagel. Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, visualised by staining with 5% H2SO4 in EtOAc from 6:4 to 3:7) to afford compound by gravity column chromatography on silica gel (gradient PE-EtOAc from 6:4 to 3:7) to afford compound

2.3. Synthesis of BGN3

BGN3 was synthesised according to the method of Zhang et al.34, whose experimental spectra were comparable. White solid. 1H NMR (400 MHz, DMSO-d6) δ 7.84 (s, 1H), 7.53 (d, J = 7.8 Hz, 2H), 7.39 (d, J = 7.8 Hz, 2H), 6.28 (bs, 2H), 5.49 (s, 2H), 4.45 (s, 2H) (Figure S1(A)). 13C NMR (100 MHz, DMSO-d6) δ 159.69, 136.80, 135.43, 128.82, 128.58, 66.44, 53.37. IR (KBr) cm⁻¹: 3638, 3462, 3322, 2799, 2132, 1424, 1257, 1163, 912, 790, 656, 514. ESI/MS: m/z [M + H⁺]² 297 (Figure S1(B)).

2.4. Synthesis of BGSN3

BGSN3 was synthesised by following the scheme in Figure S2.

2.4.1 Synthesis of 4-azido-N-(4-(hydroxymethyl) benzyl) butanamide (compound 3)

A stirred solution of compound 1 (see Figure S2; 1.176 g, 9.115 mmol, 1 eq/mol) was prepared according to the method by Huang et al.35 in DCM (30 ml), compound 2 (1.500 g, 10.939 mmol, 1.2 eq/mol; prepared according to the method by Leng et al.36 and TEA (5.08 ml, 36.460 mmol, 4 eq/mol) were added. The mixture was stirred for 10 min at room temperature, then T3P (50% molar excess) and TEA (5.08 ml, 36.460 mmol, 4 eq/mol; prepared according to the method by Kindermann et al.37 were sequentially added. The reaction was then heated at room temperature and stirred for 4 h until the complete conversion of the starting material (TLC: DCM-MeOH 9:1; Rf 4 = 0.70; RF BGSN= 0.55), then quenched by slow addition of BRINE and extraction with EtOAc. After drying (Na2SO4) and evaporation, the residue was purified by gravity column chromatography on silica gel (gradient DCM-MeOH from pure DCM to 20:1) to afford BGSN3 as a white solid (413 mg, 67%). 1H NMR (400 MHz, DMSO-d6) δ 12.48 (bs, NH purine 1H), 8.43 (t, J = 5.9 Hz, 1H), 7.85 (s, 1H), 7.49 (d, J = 7.7 Hz, 2H), 7.30 (d, J = 7.8 Hz, 2H), 6.31 (bs, NH2 purine 2H), 5.50 (s, 2H), 4.30 (d, J = 5.9 Hz, 2H), 3.36 (t, J = 6.8 Hz, 2H), 2.26 (t, J = 7.4 Hz, 2H), 1.81 (p, J = 7.1 Hz, 2H) (Figure S4(A)). 13C NMR (100 MHz, DMSO-d6) δ 171.38, 159.91, 159.69, 155.23, 139.48, 137.90, 135.31, 128.60, 127.34, 113.57, 66.59, 50.36, 41.95, 32.23, 24.58. IR (KBr) cm⁻¹: 3647, 3484, 3379, 3282, 2794, 2100, 1580, 1403, 1280, 1163, 938, 835, 789, 635, 553. ESI/MS: m/z [M + H⁺]² 382 (Figure S4(B)).

2.5 Plasmin and protein purification

The cloning procedures in the pQE31 expression vector (Qiagen, Germany) were similar for both proteins. In particular, the pSNAP-tag(m) Vector was used as a template to amplify the DNA fragment relative to the SNAP-tag gene, by using QE_SNAP-Fwd/ QE_SNAP-Rev oligonucleotides pairs (5'-ATGGCCAGATCCAA TGACAAAAGCTTAACCCAGCCCAG GCTTGCCCA G-3'; BamH I and Hind III sites, respectively, are underlined). Afterwards, the resulting fragment and the pQE31 vector were digested with BamH I and Hind III restriction enzymes and ligated, leading to the final pQE-SNAP plasmid. The final SNAP-tag protein was expressed with an extra N-terminal aminocidic sequence, comprising a His6-tag (MRGSHHHHHHTDP-). The ligation mixture was used to transform E. coli KRX competent cells and positive colonies were confirmed by PCR and DNA sequence analyses.

H² was cloned as previously described22. SNAP-tag² and H² proteins were expressed in E. coli ABLE C cells, grown at 37 °C inuria—Bertani (LB) medium supplemented with 50 mg/l kanamycin and 100 mg/l ampicillin. The protein expression was induced with 1 mM isopropyl-thio-β-D-galactoside (IPTG) at an absorbance value of 0.5–0.6 A600 nm. After overnight growth, cells were collected and resuspended 1:3 (w/v) in purification buffer (50 mM phosphate, 300 mM NaCl; pH 8.0) supplemented with 1% Triton X-100 and stored overnight at −20 °C. Subsequently, the biomass was treated in ice with lysozyme and DNAse for 60 min and then sonicated. After centrifugation of the biomass, the supernatant was dialysed against phosphate-buffered saline (PBS 1 x, 20 mM phosphate buffer, NaCl 150 mM, pH 7.3). Pooled fractions were concentrated and protein purification was confirmed by SDS-PAGE analysis. Aliquots were finally stored at −20 °C.
Competitive inhibition assay was performed as described 23,38. Briefly, by using a fixed concentration of the fluorescent SVG (5 mM) and enzymes (5 mM), an increasing amount of guanine-derivatives (0–2 mM) was added to the mixtures. The reactions were incubated for 30 min at 25 °C and 50 °C for SNAP-tag® and H5 respectively, and loaded on SDS-PAGE. Subsequently, fluorescent bands were measured by gel-imaging on a VersaDoc 4000™ system (Bio-Rad), by applying a blue LED/530 bandpass filter. Obtained data were finally plotted by Equation (1),

$$RF = \frac{100\%}{1 + \left(\frac{[I]}{[S]}\right)}$$

where RF is the obtained Relative Fluorescence, [I] and [S] are the concentration of the inhibitor and the substrate, respectively, and finally the IC50 is the concentration needed to reduce by 50% the fluorescence intensity of the protein band.

We evaluated the activity of SNAP-tag® and H5 enzymes on BGN3 and BGSN3 by the afore-mentioned IC50 method (Figure S5(A,B)) and other O6-guanine-derivatives (Table 1).

### Table 1. Substrate specificity of SNAP-tag® and H5 by competitive inhibition method (IC50) by using SVG as substrate, and second order rate constant of the enzymatic reaction of these protein-tags only on the BGSN3 substrate.

| Structure | Name  | IC50 (μM) | $k^a$ (s⁻¹ M⁻¹) | IC50 (μM) | $k^b$ (s⁻¹ M⁻¹) | Note   |
|-----------|-------|-----------|-----------------|-----------|-----------------|--------|
| SVG       | –     | $2.8 \times 10^4$ ¹ | –               | $1.6 \times 10^4$ ¹ | (14,24) |
| BG        | 36.8 ± 5.6 | –   | 10.1 ± 1.0 | –      | This work       |
| SCB       | 2.1 ± 0.5   | –   | 4.4 ± 0.8   | –      | This work       |
| BGN3      | 15.6 ± 0.3  | –   | 23.5 ± 1.0  | –      | This work       |
| BG430     | ND       | –   | ND           | –      | This work       |
| BGPA      | 86.0 ± 6.7 | –   | 14.3 ± 1.9  | –      | This work       |
| MGPA      | –        | –   | 268.9 ± 19.1 | –      | This work       |
| BGSN3     | 17.8 ± 1.1 | 4.64 ± 1.04 × 10⁵ | 10.0 ± 0.7 | 1.40 ± 0.47 × 10⁴ | This work |

For each compound, the guanine moiety is drawn in black and the chemical group conjugated to the benzyl ring in blue. The fluorescein moiety of the SVG is in green, whereas SCB differs from the other derivatives by the presence of a benzyl group (in red). Azide group is conventionally coloured in magenta.

¹Reaction rates at 25 °C; ²this value was obtained by using a BG-fluorescein substrate (BG-FL) very similar to SVG; ³not determined; ⁴this molecule is a O6-methylguanine derivative; ⁵competitive assay for H5 was performed at 65 °C.

### 2.6 AGTs’ substrate assay by competitive inhibition method

Competitive inhibition assay was performed as described 23,38. Briefly, by using a fixed concentration of the fluorescent SVG (5 mM) and enzymes (5 mM), an increasing amount of guanine-derivatives (0–2 mM) was added to the mixtures. The reactions were incubated for 30 min at 25 °C and 50 °C for SNAP-tag® and H5 respectively, and loaded on SDS-PAGE. Subsequently, fluorescent bands were measured by gel-imaging on a VersaDoc 4000™ system (Bio-Rad), by applying a blue LED/530 bandpass filter. Obtained data were finally plotted by Equation (1).

### 2.7. In vitro Huisgen Cu(I)-catalysed cycloaddition reaction

The Huisgen chemical reaction was evaluated on SNAP-tag® and H5 previously incubated with BGN3 and BGSN3. An opportune amount of purified proteins was incubated within an equimolar ratio of these substrates for 60–120 min at 25 °C and 37 °C respectively, to ensure the complete enzymatic labelling reaction. Later, we performed the subsequent cycloaddition using 5 μM of an alkyne-derivative of the fluorescein (BDP FL alkyne), in the
presence of copper (1 mM), TCEP (1 mM), TBTA (0.1 mM) and, where indicated, of SDS (0.05%). Finally, mixtures were loaded on SDS-PAGE and analysed as described in Section 4 (Figure S5(C,D)).

2.8. Molecular modelling

All molecular modelling studies were performed on a Tesla workstation equipped with two Intel Xeon X5650 2.67 GHz processors and Ubuntu 14.04 (http://www.ubuntu.com). The protein structures and 3D chemical structures were generated in PyMOL (The PyMOL Molecular Graphics System, version 2.2.3, Schrödinger LLC, 2019).

2.9. Molecular dynamics (MD) simulation

The MD simulations were carried out using the Desmond simulation package of Schrödinger LLC (Schrödinger Release 2019–1: Desmond Molecular Dynamics System; D. E. Shaw Research: New York, NY, 2019; Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2019). The X-ray structure of the H5 covalently bound to SVG was used in this study, entry code 6GA029, water molecules were removed, and all hydrogen atoms and charges were added. The NPT ensemble with the temperature of 300 K and a pressure 1 bar was applied in all runs. The simulation length was 100 ns with relaxation time 1 ps. The OPLS-2005 force field parameters were used in all simulations40. The long-range electrostatic interactions were calculated using the particle mesh Ewald method41. The cut-off radius in Coulomb interactions was 9.0 Å. The water molecules were explicitly described using the simple point charge model42. The Martyna–Tuckerman–Klein chain coupling scheme43 with a coupling constant of 2.0 ps was used for the pressure control and the Nosé-Hoover chain coupling scheme44 for the temperature control. Non-bonded forces were calculated using an r-RESPA integrator where the short-range forces were updated every step and the long-range forces were updated every three steps. The trajectory sampling was done at an interval of 1.0 ps. The behaviour and interactions between the ligands and protein were analysed using the Simulation Interaction Diagram tool implemented in the Desmond MD package. The stability of MD simulations was monitored by looking at the RMSD of the ligand and protein atom positions in time.

2.10. Determination of the rate constants of the chemoenzymatic labelling approach

Rate constants of the enzymatic reactions with the only BGSN3 were determined by the method of Gautier et al.14. In this case, purified proteins (5 μM) were incubated with the substrate (5 μM) in PBS 1× buffer at 25 °C. Aliquots were taken at different times, the reactions were immediately stopped in Tris buffer and incubated for 10 μM of Cy5 DBCO fluorophore and placing tubes on ice.

Rate constants for the chemical reaction needed for the preliminary achievement of the clickable-SNAP and clickable-H2 with BGSN3, which was obtained by the aforesaid protocol, in order to get the complete labelling. Then, to each aliquot of 5 μM of clickable proteins, 20 μM of DBCO-PEG4-Fluor 545 fluorophore was added. At different times, an excess of sodium azide (NaN3, 300 mM) was immediately added to each aliquot and then placing tubes on ice, in order to stop the click reaction between the azide group on the BGSN3 and the DBCO-PEG4-Fluor 545 molecule.

2.11. In vitro Huisgen copper-free cycloaddition reaction with different DBCO-fluorophores

For the copper-free click reaction, aliquots of 5 μM of each clickable-protein were incubated for 60 min at room temperature in the dark with 5 μM of fluorescent DBCO-derivative substrates (BDP FL DBCO, Cy5 DBCO, and DBCO-PEG4-Fluor 545) in a total volume of 10 μL of PBS 1× buffer (Figure 4 and Figure S6). The reactions were finally stopped in Leammli Buffer 3×, loaded on SDS-PAGE, and analysed as described in Section 4, by applying a blue LED/530 bandpass filter, red LED/695 bandpass filter and green LED/605 bandpass filter as excitation/emission parameters for each DBCO-fluorophores, respectively. The click reaction was also performed on 5 μM of both the enzymes, but in the presence of an EcCFE diluted in PBS 1× buffer.

2.12. Procedure for protein immobilisation on bio layer interferometry (BLI), by following the chemoenzymatic approach

OctetRED96TM (Fortebio, Fremont, CA) was used to immobilise specifically SNAP-tag® and H2 with the chemoenzymatic approach (Figure S5(A,B)). Samples and reaction buffers were located in black 96-well plates (OptiPlate-96 Black, Black Opaque 96-well Microplate, PerkinElmer, Billerica, MA) in a maximum reaction volume of 300 μL per well with 800 rpm shaking for each step. For the immobilisation procedure, AR2G sensors were first wetted in 200 μL of pure water for at least 15 min, followed by an equilibration step (3 min) in acetate buffer 0.1 M, pH 5.0. Afterwards, they were activated with 20 mM 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC)/20 mM N-hydroxy-sulfo-succinimide (sulfo-NHS) mixture in acetate buffer (60 min) and covered with 2 mM propargyl-PEG3-amine bifunctional linker (BroadPharm, San Diego, CA) in Loading step (20 min). To avoid the presence of any free amine groups on the biosensors, a Blocking step with Ethanolamine 1 M (30 min) was performed. Subsequently, a Washing step (15 min) with water and an Equilibration step in click-reaction buffer (15 min) are followed.

During the afore-described procedure, proteins were labelled with BGSN3. Finally, the immobilisation step for each sample via Huisgen reaction was carried out at 30 °C for 80 min, followed by a Washing step (20 min), in order to remove all the unbound molecules. This procedure was the same in the presence of the EcCFE. All measurements were performed in triplicates.

2.13. Permeability of eukaryotic and prokaryotic cells to BGSN3

HEK293T cells were maintained at 37 °C with 5% CO2 in Dulbecco’s Modified Essential Medium (Invitrogen, Carlsbad, CA) supplemented with 10% Foetal Bovine Serum (FBS) (Invitrogen) and 100 U/ml Penicillin/Streptomycin (Roche, Switzerland). HEK293T cells were transfected with SNAP-tag® plasmid by using
Lipofectamine 2000 (Invitrogen) following manufacturer’s protocol. The treatment with BGSN3 were performed, at the concentration and time indicated for each experiment. Twenty-four hours after transfection, we treated cells with BGSN3 for 2 h at different concentrations ranging (from 1 to 25 μM), directly dissolving the compound in complete culture medium. Then cells were harvested, washed with PBS 1× buffer and lysed with 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100

Figure 2. Reaction rates of the chemo-enzymatic approach. Pseudo-first-order reaction of protein-tags for (A) the enzymatic reaction with BGSN3 (see k values also in Table 1), and of clickable-tags for (B) Huisgen reaction with DBCO-PEG4-Fluor 545 (see values in the main text). Values given are an average of three independent measurements. The reaction scheme was an exemplification of Figure 1(B) in the main text. Data are represented as mean ± SEM.

Figure 3. Molecular modelling on H5 with BG-azides. (A) RMSD of the atomic positions for the compound BGN3 (Lig fit Prot, in red) and the protein H5 (Ca positions, in blue) of the 100 ns molecular dynamics simulations using Desmond package. (B) A timeline representation of the interactions and contacts (H-bonds, Hydrophobic, Ionic, Water bridges). (C) RMSD of the atomic positions for the compound BGSN3 (Lig fit Prot, in red) and the protein H5 (Ca positions, in blue) of the 100 ns molecular dynamics simulations using Desmond package. (D) A timeline representation of the interactions and contacts (H-bonds, Hydrophobic, Ionic, Water bridges). (E) Solvent Accessible Surface Area (SASA) of BGN3/H5 (in orange) and BGSN3/H5 (in cyan) complexes over the MD simulation time (mean values are depicted as dot lines). Frames of H5-probe complexes with lower (F, H) and higher (G, I) SASA value for BGN3 (F, G) and BGSN3 (H, I), respectively.
supplemented with complete protease (Roche, Switzerland) and phosphatase (SERVA Electrophoresis, Germany) inhibitors. Afterwards, transfected cells were treated with a fixed concentration of BGSN3 (10 μM) at different time points (from 30 to 120 min). Again, HEK293T cells were washed and lysed as described before. To confirm the reaction with BGSN3, the same amount of protein extract (0.91 μg/mL for each sample) was incubated for 30 min at 25°C with SVG. Subsequently, proteins were loaded on SDS-PAGE and analysed by gel-imaging on a VersaDoc 4000™ system (Bio-Rad), by applying a blue LED/530 bandpass filter (Figure 6).

For flow cytometry analysis, HeLa cells were seeded in 24-well plates and transfected with SNAP-tag® plasmid by using Lipofectamine 2000 (Invitrogen, USA) following manufacturer's protocol. Twenty-four hours after the transfection, cells were treated with 25 μM BGSN3 for 1 h, and the excess of the substrate was washed out by 2 × 15 min, followed by 1 × 30 min washes. Cells where then treated with 2.5 μM BDP FL DBCO for 30 min and unbound fluorophore was removed by following the same procedure performed for the BGSN3. All treatments and washes were performed at 37°C in a complete culture medium. Lastly, cells were harvested by trypsinization, and fluorescence was measured using FACS CANTO II instrument. The analysis was performed on live singlet cells using FlowJo software (Figure S7A).

E. coli ABLE C strain was transformed with SNAP-tag® plasmid and protein expressed as previously described. After overnight
Figure 5. Covalent immobilisation of clickable-tags on the BLI sensor. (A) Covering of the BLI sensor with a bi-functional linker, exposing alkyne groups for the Huisgen cycloaddition reaction; (B) reaction of the SNAP-tag\(^*\) with BGSN3; (C) chemo-enzymatic SNAP-tag\(^*\) immobilisation on BLI. The alkyne-covered sensor (silver cylinder) was immersed in wells containing the buffer (in black), the free SNAP-tag\(^*\) (in blue) and the clickable-SNAP (in magenta); (D) column chart relative to the BLI immobilisation of purified protein-tags alone (black-bordered bars) or in the presence of BGSN3 (magenta-bordered bars). Filled magenta bars represent the BLI immobilisation using the EcCFE upon heterologous expression of protein-tags. Standard deviations were obtained from three independent experiments. Data are represented as mean ± SEM.
growth, samples of 2 ml were treated with 100 μM of **BGSN3** for 2 h at 25 °C and then collected by centrifugation at 2000 × g. Cell pellets of 0.05 g were resuspended 1:3 (w/v) in PBS 1× supplemented with 1% Triton X-100 and subjected to cell lysis, by applying 5 cycles of freeze-thawing. After a centrifugation at 13,000 × g, the supernatants containing the protein extract were incubated 30 min at 25 °C with **SVG**, and proteins were loaded on SDS-PAGE. Finally, fluorescent bands were analysed by *gel-imaging* techniques (Figure S7(B)).

### 3. Result and discussion

#### 3.1. Substrate specificity of AGTs on BG-based substrates

Following the irreversible reaction shown in Figure 1, we evaluated the activity of two enzymes in our possession on several O6-guanine-derivatives (Table 1). Because most of them are non-fluorescent compounds, we performed an AGTs’ competitive inhibition assay by using the fluorescein-derivative SNAP-Vista® Green as substrate (**SVG**), as previously described. Briefly, the reaction of an AGT with **SVG** led to a fluoresceinated protein, which can be visualised as a fluorescent band in *gel-imaging* analysis after SDS-PAGE. The presence of increasing amounts of a non-fluorescent competitor in the reaction causes a decrease of the fluorescent signals, which can be measured and plotted for the IC50 values determination. As shown in Table 1, SNAP-tag® and H2 displayed different behaviours versus these competitors, without any rationale for the dimension and/or polarity of the conjugated chemical groups. While SNAP-Cell® 430 (SC430) completely lost the competition with **SVG**, both the enzymes are extremely active on the SNAP Cell® Block (SCB), displaying the lowest IC50 value measured. This result was expected, because SCB has a structure very similar to the Lomeguatrib, one of the most efficient inhibitors of the hMGMT protein, employed in the cancer treatment in combination with alkylating agents-based chemotherapeutics.

In general, all commercially available products used (**SVG**, **SCB**, **BG430**, and **BG-PEG-NH2**, **BGPA**) are good substrates for the SNAP-tag® and H2 enzymes, completing their labelling reaction in few hours (data not shown). However, based on our results, the choice of the chemical group to be conjugated to the O6-guanine for customized substrates may present risks, with consequent decreases in the reaction rate for these protein-tags. This was the case of methyl-guanine-PEG-NH2 (**MGPA**), which is an O6-methyl-guanine derivative, used for the immobilisation of SNAP-tag® on nanoparticles. The latter is not a preferred substrate, probably

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**Table 1:** Eukaryotic permeability to BGSN3. SDS-PAGE analysis by *gel-imaging* and coomassie staining of HEK293T cell lysates. After BGSN3 in medium treatment, lysates were incubated with **SVG**.

| **SNAP-tag®** | **HEK293T cells** | **HEK293T / pSNAP(m) ce** | **BGSN3** | **SVG** |
|---------------|-------------------|--------------------------|----------|--------|
|               | +                 | +                        | -        | +      |
|               | -                 | +                        | +        | +      |
|               | -                 | -                        | +        | +      |

**Figure 6.** Eukaryotic permeability to BGSN3. SDS-PAGE analysis by *gel-imaging* and coomassie staining of HEK293T cell lysates. After BGSN3 in medium treatment, lysates were incubated with **SVG**.
because of the absence of the benzyl ring, which leads to complete labelling of the SNAP-tag\(^\circ\) and \(H^2\) after over-night incubation at 4 °C\(^{39}\) and 65 °C (data not shown), respectively.

### 3.2. In vitro enzymatic reaction of engineered AGTs with BG-azide substrates

Recent studies were focussed on the synthesis of alternative “BG-building blocks,” which offer the opportunity to produce SNAP-substrates by following easier and faster protocols: an alkyn substituted \(O^\circ\)-BG was employed in the synthesis of compounds by the Huisgen cycloaddition with azide-based fluorescent probes\(^{19}\) or, inversely, by using the \(O^\circ\)-BG-N\(_3\) (BGN3, Figure S1) for the conjugation with alkyn-based chemical groups\(^{34}\). We evaluated the enzymatic reaction of the \(H^2\) and the SNAP-tag\(^\circ\) directly on BGN3 and a synthesised BG-derivative containing a benzyl ring opportunely spaced from the azide group (BGSN3, Figure S4); after the reaction, no fluorescent signal was obtained on SDS-PAGE gel-imaging upon the addition of SVG (Figure S5(A,B)). This indicates that the catalytic cysteine was completely blocked by the benzyl-azide moiety, impeding the access of the fluorescent substrate to the active site. Compared to the classical BG-derivatives, these protein-tags showed a reasonable activity on both these BG-azides, as resulted by the calculated IC\(_{50}\) (Table 1 and Figure S5(A,B)).

After the enzymatic reaction of \(H^2\) with BGN3 and BGSN3, we performed the subsequent cycloaddition using an alkyn-derivative of the fluorescein (BDP FL alkyne); however, the chemical reaction was less efficient using the former substrate (Figure S5(C), lane 2). In this case, the complete fluorescein labelling of the protein was achieved only in the presence of a small amount of SDS during the cycloaddition step (lane 3), suggesting that the protein is still folded after the enzymatic reaction and the azide is hidden in the active site core. The addition of the denaturant could have slightly opened the protein structure, favouring a better exposure of the azide group to the solvent, and allowing the click reaction to occur.

On the contrary, using BGSN3 as substrate, the labelling of both the enzymes was comparable to the classical reaction with SVG without any denaturing agent, likely the longer spacer of BGSN3 could sufficiently move away from the azide group from the protein surface for the Huisgen reaction (Figure S5(D), lanes 2 and 4). From now on, experiments were only performed by using the longer BG-azide. We first calculated the rate of the enzymatic reaction, demonstrating that both protein-tags show a high catalytic activity comparable to the commercial BG-derivatives currently used (Figure 2(A) and Table 1), also indicating that the complete protein labelling in less of an hour can be performed\(^{13,14,24}\).

### 3.3. Molecular modelling on the \(H^2\) with BG-azides

BGN3 and BGSN3 differ in length since the chemical spacer between the benzyl ring and the active azide makes the latter potentially more prone to the labelling reaction. It could be assumed that this aspect alone influences the availability of the azide moiety to react. However, proteins are not a static system, the amino acids side-chain movements could mask the azide and prevent the “click” chemistry reaction. The covalent complexes of these compounds with \(H^2\) were analysed with Molecular Dynamics (MD) simulations using the Desmond package (see Experimental Section). The complexes were simulated for 100 ns at 300 K using a standard protocol. The protein structure has been stabilised, as shown in the RMSDs for both the IDO1 C\(_x\) and the ligand (Figure 3(A,C)). The MD results were analysed in terms of Solvent Accessible Surface Area (SASA) of the compounds: more time the compounds are exposed to the solvent, the higher is the possibility to react\(^{35}\). In Figure 3 is reported the fluctuation of the SASA values over the simulation time together with the structure model of the \(H^2\) protein in complex with BGN3 and BGSN3, respectively. The former is less exposed to the solvent with a SASA value of 32.967 ± 18.573 Å\(^2\) compared to BGSN3, which shows a higher SASA value 68.302 ± 32.455 Å\(^2\). This simulation confirmed our biochemical data, proposing the BG-derivative with the spacer as a better substrate for our chemo-enzymatic approach.

### 3.4. Specificity and versatility of the chemo-enzymatic reaction

The \(O^\circ\)-BG-based BGSN3 is a good substrate for the two protein-tags used (Table 1 and Figure 2(A)) and offering the advantage to sufficiently expose the azide group for the Huisgen reaction. This was the starting point to examine: (i) the labelling efficiency of the clickable-SNAP and clickable-\(H^2\) by using different DBCO-based fluorophores; (ii) the specificity of the “click” reaction.

Upon the reaction with BGSN3, all cycladdition reactions with three different DBCO-based fluorophores were complete in ca. 30–45 min in PBS 1 x buffer (Figure 4, lanes 2–4), with a protein-labelling as efficient as the enzymatic reaction using the sole SVG (lane 1). We quantitatively evaluated the rate (\(k\)) of the click reaction by using the DBCO-PEG4-Fluor 545 fluorophore: as expected, both the clickable-tags were labelled with the same efficiency (1.83 ± 0.41 × 10\(^{3}\) s\(^{-1}\) M\(^{-1}\) for SNAP-tag\(^\circ\)\(^{53}\), 1.54 ± 0.39 × 10\(^{3}\) s\(^{-1}\) M\(^{-1}\) for \(H^2\)), demonstrating that the chemical reaction is sufficiently fast and independent from the tags (Figure 2(B)).

Concerning the specificity, we added a crude protein extract from Escherichia coli ABLE C (EcCFe), without any AGT activity at the gel-imaging analysis (Figure 4, lane 5). In this context, the only presence of the free protein-tag and the DBCO-fluorophore also did not result in any fluorescent signal (lanes 6), whereas the previously purified clickable-SNAP (lane 7), as well as its free form in the presence of BGSN3 (lane 8), was specifically able to complete the chemo-enzymatic reaction, giving an evident fluorescent signal. The high specificity of our approach was also confirmed by using the \(H^2\) enzyme, which displays a better labelling reaction than the mesophilic SNAP-tag\(^\circ\) (Figure S6). Probably, something in the extract might impede SNAP-tag\(^\circ\) activity. These results clearly demonstrated the high efficiency of our chemo-enzymatic approach for the labelling of both the protein-tags used.

### 3.5. Application to the bio layer interferometry

The possibility to apply the SNAP-tag\(^\circ\) technology to the Surface Plasmon Resonance (SPR) for the covalent immobilisation of a protein of interest was first explored by the group of Kai Johnsson\(^{37}\), followed by other groups with the same substrate\(^{51}\) or a biontin BG-derivative\(^{52}\). Their approaches, again, required preliminarily the synthesis and the purification of a compatible substrate to cover the sensor chip surface. We used, instead, the BGSN3 substrate for the immobilisation of the SNAP-tag\(^\circ\) directly on an alkyn-derived sensor chip of the bio layer interferometry (BLI) equipment, as shown in Figure 5. This technique is more advantageous with respect to the SPR because: (i) it needs a smaller amount of sample, making it more compatible to higher throughput (the capacity of running up to 96 samples in a parallel); (ii) the possibility to reuse samples, and (iii) the total independence from any microfluidic issues.
Given the lack of any available BLI alkyne-derived sensors, we first activated the AR2G type by a bi-functional linker (propargylPEG 3-amine) in order to expose an alkyne group on the surface (Figure 5(A)). This modified protocol provides first the coating of the sensor tips with alkyne groups (approx. 80 min), during that the reaction between the protein-tag and BGSN3 inside the 96-wells rack takes place (Figure 5(B)). Only the contemporary presence of the clickable-SNAP and the alkyne-coated sensor led to a measurable response (Figure 5(C)). After washing procedures, the signal did not significantly drop-down, confirming the covalent reaction between the protein and the sensor. We successfully achieved results with both the enzymes, although temperature and times of the enzymatic reaction on BLI (30 °C) favoured the SNAP-tag® respect to the thermophilic H. pyruvatic. Furthermore, in ECFEs where both the enzymes were expressed, a specific and efficient immobilisation on BLI sensor tips occurred (Figure 5(D)), although the SNAP-tag® displayed a lower labelling efficiency in the ECFE, as expected (compare lane 8 in Figure 4 and Figure S6). As for other techniques, this specific surface immobilisation of SNAP-tag® gives the opportunity to perform a directly on-chip purification of a tagged-POI from a crude lysate, without any purification step, in an indirect manner, which favours a better orientation of the POI for its biological activities.

3.6. Permeability of eukaryotic and prokaryotic cells to BG-azides

One of the major applications of the SNAP-tag® technology concerns the field of cell biology, where detecting fluorescent-tagged-POIs in living cells represents an important tool to study protein functions and locations. To test our chemo-enzymatic approach, we first investigated the permeability of BGSN3. Lysates of HEK293T cells pre-treated with BGSN3 were then incubated with the SVG substrate: the absence of any fluorescent signal by gel-imaging only in BG-azide treated lysates demonstrated that the internalisation of BGSN3 was fast (ca. 30 min; Figure 6, lane 3) and at concentrations comparable with commercial cell biology BG-substrates (in the range of <5 µM; Figure 6, lane 8). Preliminary experiments by FACS analysis confirmed that the in vivo cycloaddition between BGSN3 and the BDP-FL DBCO occurred (Figure S7(A)). This was also confirmed for E. coli bacterial cells (Figure S7(B), lane 2).

4. Conclusions and perspectives

We developed an innovative modification of the SNAP-tag® technology, in order to overcome times and costs relative to the production and the utilisation of commercial or purified customised DG-derivatives. Although they are complementary in terms of catalytic activity as for the SNAP-tag®, as well as for the other AGTs, the risk of lowering the catalytic activity of these tags with customised DG-derivatives should not be underestimated (Table 1). We started by the knowledge that: (i) self-labeling protein-tags are still folded and enough stability in their benzylated form after the enzymatic reaction; (ii) the Huisgen cycloaddition is extremely versatile, fast and specific. Recently, the latter was used for the entrapment of catalytic activities by azide-based pseudo-substrates in a well-known powerful method, the in vivo activity-based protein profiling (ABPP). For these reasons, a chemo-enzymatic approach (Figure 1(B)) with an opportunely selected azide-based BG-substrate (BGSN3) was set up: the efficient exposition of the azide outside the protein surface allows the reaction with a huge number of commercially DBCO-based molecules, keeping high the specificity in the presence of in vitro “perturbing” proteins (like in cell lysates) and the in vivo labelling of expressed SNAP-tag® in eukaryotic cells. Finally, BGSN3 proved to be a good substrate for the direct immobilisation of these tags on solid surfaces. We demonstrated that splitting the SNAP-tag® reaction into two fast steps, as experimentally measured (Figure 2(A,B)), does not affect the overall rate and efficiency of the protein labelling, thus opening new perspectives and widening the applications of this powerful biotechnology.

Authors’ contributions

Conceptualisation, G.P. and A.Mi.; Methodology, R.Me., G.P.; Investigation, R.Me., D.C. and M.C.; Formal Analysis, C.M., R.Mi. and A.Ma.; Validation, R.Me., R.Ma., A.Mi. and G.P.; Writing – Original Draft, R.Me. and G.P.; Writing – Review & Editing, C.M., F.R. and A.V and G.P.; Funding Acquisition, A.V. and G.P.; Resources, R.Me, R.Ma. and D.C.; Supervision, A.L., A.Mi. and G.P.

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Disclosure statement

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