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ARTICLE

"Click and go": simple and fast folic acid conjugation

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Folic acid targeting by functionalization the terminal γ-carboxylic acid is one of the most important strategies to selectively deliver chemotherapeutics and dyes to cancer cells which overexpress folate receptors. However, conjugation efficiency to folic acid is limited by its unique solubility and by selectivity issues imposing the need for expensive preparative reverse-phase chromatographic purifications to isolate γ-folate conjugates. Herein is provided a novel synthetic tool for the synthesis of new folic acid conjugates with excellent γ-purity based in strain-promoted alkyne/azide cycloadditions with a γ-folate-cyclooctyne conjugate **3**. To demonstrate the potential of this methodology several new folate conjugates were synthesized with high γ-purity and without using any type of chromatographic purification by reacting conjugate **3** with several fluorescent probes, polymer and siliceous materials bearing azide. In addition, the cycloaddition reaction between conjugate **3** and azido-derived fluorescent dye was successfully performed in cellular media leading to an increase of fluorescence in the cells which over-express folate receptors (NCI-H460).

Introduction

In the last decades, folic acid has been employed to actively target cancer cells constituting a very active field of research in medicinal chemistry. This small vitamin is crucial for the normal cell functioning and their receptors were found to be over-expressed in a considerable percentage of cancer cell lines.[1-2] Nowadays, a hand-full of small folic acid conjugates with drugs or fluorescent probes were described and are being evaluated in clinical trials.[3-5] Apart from these developments, the synthetic tools available for conjugation into folic acid are relatively underdeveloped and remain highly challenging.[6-8]

This vitamin is structurally constituted by pteroic acid covalently bound to a glutamic acid residue. However, only the γ-conjugates have medicinal relevance, since they have higher affinity toward the receptors compared with α-conjugates.[9] Recently, the FRα protein was isolated and crystalized providing important insights into the interaction between the receptor and folate.[10] Folic acid docks with the pterin head buried in the receptor pocket, being stabilized by numerous hydrogen bridges and hydrophobic interactions. Even the glutamate residue is stabilized by six hydrogen bonds, four of which with the α-carboxylic acid. These observations clearly indicate that only γ-folate conjugates can retain affinity toward the receptor.

Fortunately, the γ-conjugates are intrinsically obtained as the major product (from 55 to 90 % selectivity[7,9,11-15]) using carbodiimide chemistry. The separation between low molecular weight folate conjugates by precipitation (from α-conjugates, bi-conjugates or unreacted folic acid) becomes troublesome as these molecules are only soluble in DMSO in their neutral form. Therefore their separation requires in general tedious and expensive preparative reverse phase chromatography.[7,9,16-18] When the conjugation is performed in macromolecules or heterogeneous materials the conjugate acquire the properties of the material,[18-27] allowing for non-conjugated folic acid to be easily removed, while α-conjugates and bis-conjugates will remain inseparable. Svenson raised the issue of introducing targeting ligands into supramolecular structures that also display passive targeting. This type of active targeting can introduce concerns to the regulatory evaluation and approval because it adds one synthetic step that can have a negative impact the reproducibility of the synthesis and on the polydispersity of the macromolecule.[28]

In order to circumvent the limitations of direct conjugation of alcohols and amines to folic acid, Low and Fuchs have shown that it is possible to synthesize regioselectively γ-conjugates by using a folate reconstructive strategy (Scheme 1, route A). The concept is based on the conversion of folic acid into pteroic acid, by the cleavage of the glutamic acid residue,[6-29] followed by the reattachment of a previously γ-functionalized glutamic moiety. The methodology was applied to the synthesis of small folic acid conjugates with drugs and dyes bearing a hydro-soluble and bioresponsive polypeptide spacer,[5,30-35] and in the preparation of the highly soluble 2-(trimethylsilyl)ethoxy-bisprotected folic acid conjugate **1** (Scheme 1, route A).[8] Unfortunately, this methodology also required purification by reverse phase chromatography.

Amines featuring both azides and terminal alkynes have also been conjugated with folic acid in order to exploit copper-catalysed "click" chemistry.[23,36-38] Even though this strategy is more appealing than the direct amide or alcohol coupling since it is bioorthogonal, it employs a transition metal which forms stable complexes with pteridines[39-41], and is responsible for undesirable toxicity in cells.[42] More recently, Burke's group explored cyclooctyne-tetrazine cycloaddition chemistry to link drugs into a folate-cyclooctyne conjugate **2** having a PEG-SU-Lys spacer (Scheme 1, route A). Unfortunately, the preparation of the reactive conjugate relied on chromatographic separations and the cycloaddition conjugation reaction gave a mixture of regioisomers in low to good yields (28-79 % isolated yields).[43]

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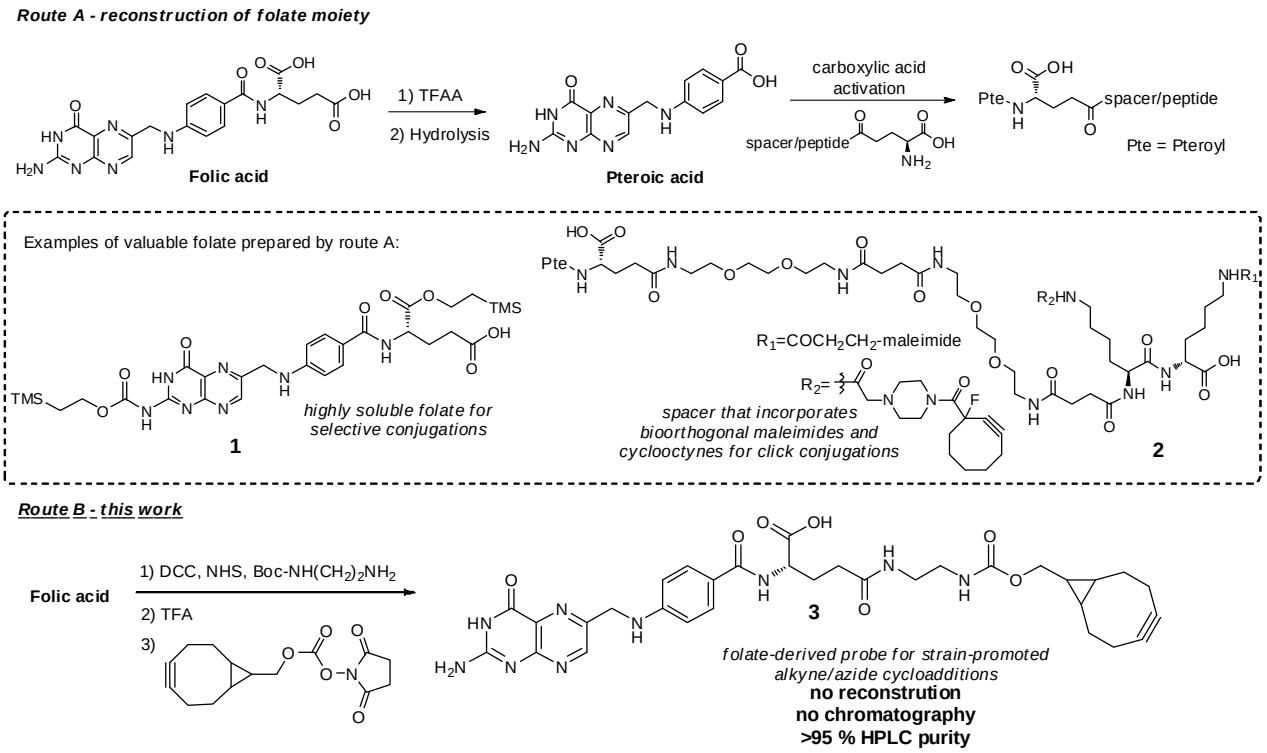
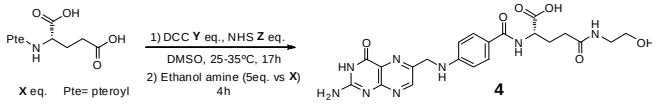


Scheme 1- General main synthetic methodologies available for folic acid conjugation and this work proposal

Therefore, we envisioned the development of a small folate conjugate that incorporates a non-metal based "clickable" functional group[42] allowing fast, selective and quantitative conjugations into folic acid. This conjugate should also have an ethylene diamine spacer in order to ensure high affinity to folate receptors.[9,44]

Hence, herein is described a new folic acid conjugation route based in strain-promoted alkyne/azide cycloadditions (spAAC)[45-46] for folate-based cancer cell targeting. The cyclooctyne BCN (BCN stands for bicyclo[6.1.0]non-4-yn-9-ylmethanol) was selected as the most appropriate clickable probe as it is commercially available, symmetrical, highly reactive (0.29 $M^{-1}s^{-1}$ in spAAC) with low lipophilicity.[47] The γ-conjugate folate-diethylenediamine-BCN **3** was synthesized in high purity employing a direct amide coupling strategy (Scheme 1, route B), without using chromatographic purifications. The procedure was demonstrated in the conjugation of folic acid to PEG, common fluorescence probes and silica-based materials.



Entry	Folic acid X eq.	DCC Y eq.	NHS Z eq.	Base (2 eq.)	Conv. (%)[a]	Select. (%)[s]
1[c]	1	1	1	-	67	88
2[c]	1	2	2	-	96	92
3[c]	2	2	1	-	48	88
4[c]	2	1	2	-	33	84
5[c]	1	2	1	-	98	87
6[d]	1	2	1	Organic bases	0-99	73-94
7[d]	1	2	1	Inorganic bases	2-85	56-87
8[d]	1	2	1	DIPEA[e]	21	94
9[d]	1	2	1	Pyridine	99	90

a) Determined by RP-HPLC (C18 Luna Phenomenex) and represents the percentage of new compounds formed relatively to initial folic acid amount; b) percentage of the γ-carboxylic amine obtained relatively to other products formed; c) 35ºC (temperature close to pyshiological for protein functionalization); d) 25ºC. e) Disopropyl ethyl amine

Table 1 - Selected examples for the optimization of folic acid γ-conjugation to ethanol amine

Results and discussion

The proposed strategy to prepare "clickable" conjugate **3** relies on a simple reaction between folate ethylene diamine with BCN-NHS mixed carbonate. The synthesis of the ethylene diaminefolate precursor is known[9,17,48-50] however no consistent information regarding the conjugation selectivity in to folic acid is available. Hence, we began to study the influence of reagent stoichiometry, temperature and basic additives (added in the initial DCC activation period) in the conjugation yield and γ-selectivity (Table 1), using ethanol amine as a surrogate for N-BOC-ethylene diamine. After an extensive screening, conjugate **4** was obtained with optimal yield and selectivity when folic acid was activated in the presence of two equivalents of DCC and NHS at 25ºC during 17 hours (for more details see Table 1 in SI). Under these conditions the γ-conjugate was obtained with 92 % selectivity (Table 1, Entry 2). Kinetic studies revealed that suboptimal conversion and selectivity were obtained for shorter reactions times (Table 2 in SI). The excess of DCC is mandatory to achieve quantitative conversions of folic acid (Table 1, Entries 1-5) while, the addition of basic additives did not impacted positively neither the selectivity nor the conversion (Table 1, Entry 6-7).[51] Gratifyingly, when the conjugation was performed with N-Boc ethylene diamine, the conjugate **5** was isolated in 73 % yield (Scheme 2) with excellent purity (over 95 % based on RP-HPLC). Treatment

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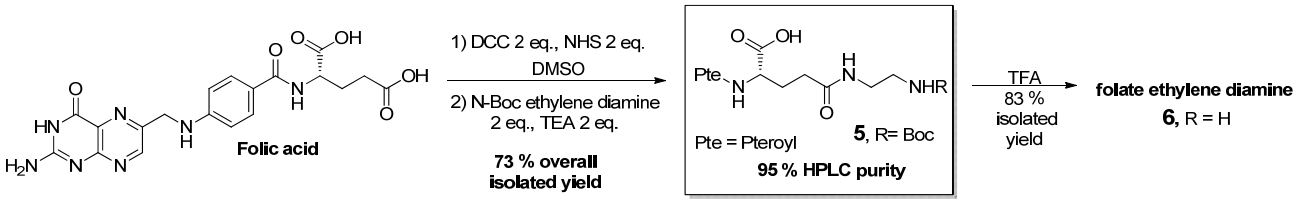
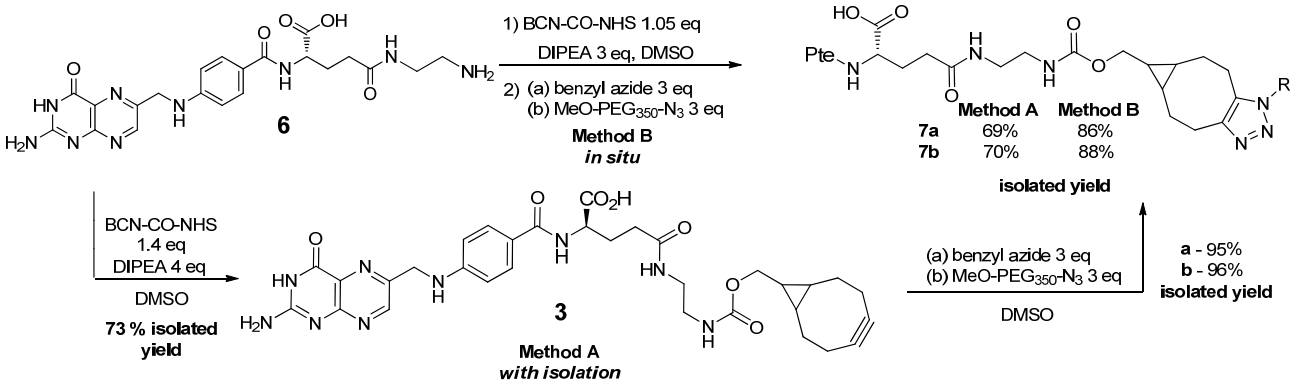


Scheme 2 -Synthetic sequence for folic acid selective activation



Scheme 3-Synthesis of conjugate **3** and its efficient conjugation into azides via strain-promoted click chemistry

with TFA for 2 hours delivered the folate N-ethylene diamine **6** in 83 % isolated yield (Scheme 2).

Subsequently, the synthesis of the desired folate ethylene diamine BCN conjugate **3** was attempted (Scheme 3), by reacting BCN-NHS mixed carbonate in the presence of DIPEA during 16 hours as reported.[52] We observed the formation of a folate-containing impurity along with the desired carbamate **3**. Such impurity was formed due to extended reaction time, since ^{1}H NMR experiments indicated that the mixed carbonate aminolysis occurs in less than five minutes yielding carbamate **3** as the single folate-derived product in quantitative yield (>95 %) (see SI Figure 1 for more details). This was further confirmed when the addition of two equivalents of benzyl azide and MeO-PEG$_{350}$-N$_3$ resulted in the complete conversion (>95%) toward the respective triazoles in 1 and 2.5 hours, respectively (SI Figure 2). Based on this data, two methodologies were developed for the preparation of new folate conjugates via copper-free click chemistry (scheme 3). In method A, folate conjugate **6** was reacted for 30 minutes with 1.4 eq. of BCN/NHS mixed carbonate in presence of 4 eq. of DIPEA yielding the desirable folate ethylene diamine BCN conjugate **3** in 73% isolated yield.

Then, the respective triazoles **7a** and **7b** were isolated in 95-96 % yield with high purity (by ^{1}H NMR) after being reacted 2 hours with 3 eq. of benzyl azide and MeO-PEG$_{350}$-N$_3$, respectively. This constitutes an overall yield up to 70% starting from folate conjugate **6** (or 42% from folic acid after 4 steps). These results confirm the effectiveness of copper-free strain-promoted click chemistry for the synthesis of new folate conjugates, easily purified from the azide excess (Scheme 3, method A).

The two-step triazole yields can be further improved up to 88 % from folate conjugate **6** (or 53 % from folic acid after 4 steps), while maintaining the same purity level, by employing method B: conjugate **3** was prepared using only 1.05 eq. of BCN/NHS carbonate and 3 eq. of DIPEA and then reacted *in situ* with only 2 eq. azides (Scheme 3, method B). It is worth mentioning that folate triazoles were synthesized with excellent γ-selectivity and high purity without using any chromatographic purification, and that conjugate **3** was stable for at least 24 hours in wet DMSO solutions. Subsequently these methods were evaluated towards the synthesis of new folic acid-derived fluorescent probes (Scheme 3). Dansyl (**c**), fluorescein (**d**), rhodamine 6G (R6G, **e**) and 7-nitro-4-aminobenzofurazan (**f**) featuring azide functionalities were synthesized and conjugated with the folic acid, to prepare a family of fluorescent conjugates that covers a broad range of the UV-Vis spectrum. Method A was employed in the conjugation of fluorescein, R6G and benzofurazan furnishing the respective triazoles with 64-68 % isolated yield, while with method B the dansyl conjugate was obtained in 70 % isolated yield (Scheme 4), with excellent purity.

Method B was also successfully applied in folic acid grafting into heterogeneous supports, like silica gel. For this purpose, flash chromatography silica gel grade was firstly treated with 3-((6-azidohexyl) thio) propyl triethoxysilane and then reacted with conjugate **3**. After 24 hours a pale yellow powder **7g** was isolated in 75 % yield (Scheme 4). In order to confirm the chemical immobilization of folic acid into the silica particles, the isolated powder was packed in a column and eluted with DMSO without any observable leaching of folates (for more details please consult SI).

As presented herein, the copper-free cycloadditions between conjugate **3** and azides constitutes a powerful synthetic tool to introduce folic acid-based targeting into polymers (exemplified by PEG), heterogeneous supports (like silica) and low-molecular weight fluorescent dyes, due to the simplicity of synthesis and isolation. This synthetic tool may also contribute to a low polydispersity of macromolecules subject to functionalization.

After, we decided to study how the photophysical properties of the dyes were impacted on conjugation into folic acid. Scheme 5 compares the absorption and emission spectra of the azido free dyes and the respective folate conjugates (obtained using μM concentrations in 1% DMSO/water). The folate conjugation lead to very little effects on the shape of the absorption and emission bands associated with transitions localized in the fluorophores. This is mostly evident when comparing **7d** and **7e** with the respective azido free dyes (Scheme 5b and c). In the aqueous mixture the emission quantum yield of the **7d** and **7e**

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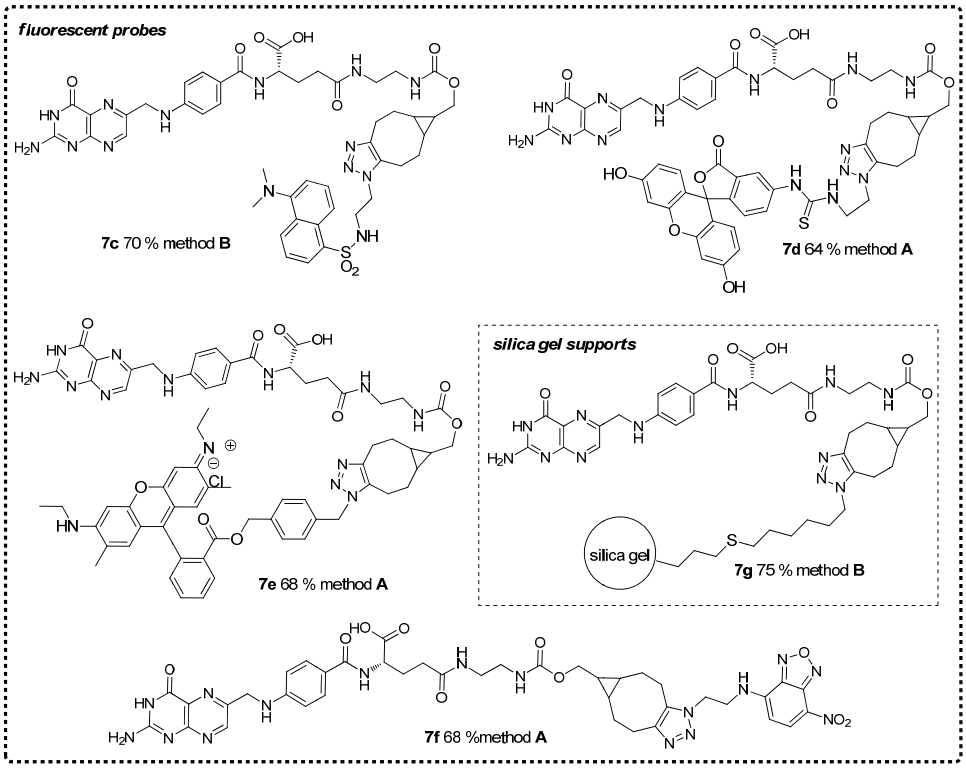
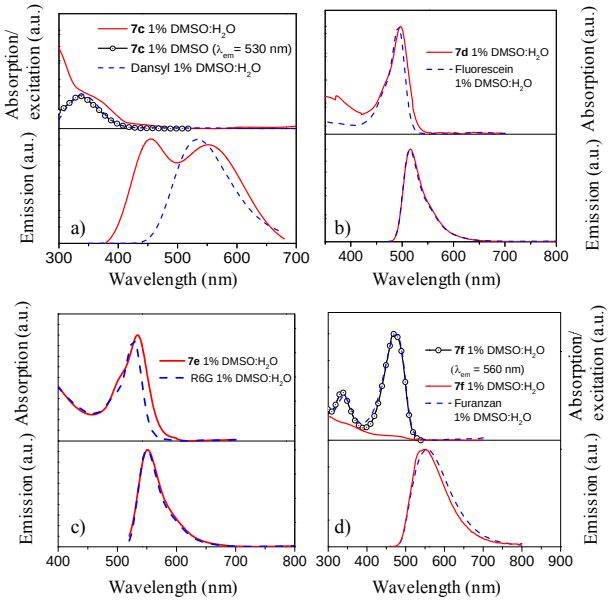


Scheme 4- Examples of folic acid conjugates prepared from **3**

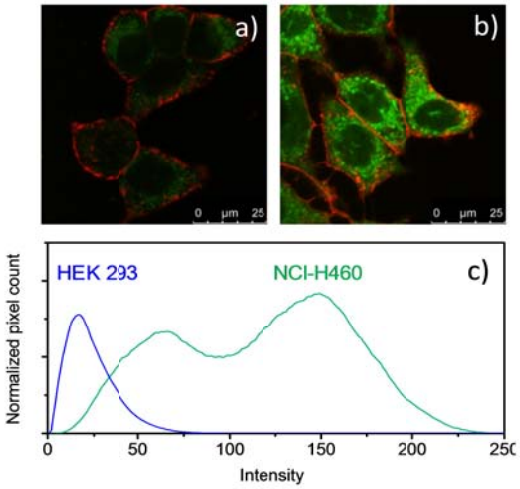
(7-9%) are about one order of magnitude lower compared with the azido free dyes.

The absorption spectra of **7c** is dominated by the contribution from the folate residue (red line in Scheme 5a), which has its lowest energy transition at about 360 nm, overlapping with the low absorption cross-section transition localized in the dansyl unit (at 340 nm). This overlap makes the **7c** conjugate a poor candidate for cell staining. The absorption spectrum of **7f** in aqueous media is also dominated by the folate residue, but the weak absorption of the benzofurazan unit is detected at 470 nm (Scheme 5d, red line). This band assignment is supported by the observation of two bands at 340 and 475 nm (typical of this dye[53]) in the corresponding excitation spectrum collected at 560 nm (red dashed line), which overlaps almost perfectly with the absorption of the free dye (red dashed line with triangles). A strong overlap is also observed between the emission of the **7f** and the free dye. The quantum yield of **7f** in the aqueous mixture is quite low (~1-2%) but, as shown by the emission spectra in Scheme 5d, it is possible to excite selectively the dye and separate its emission from emission of the folate residue (observed at 455 nm in scheme 5a). The decrease in the absorption of **7f** observed in aqueous solution relatively to DMSO suggests the formation of aggregates.[54]



Scheme 5 – Comparison between the normalized absorption and emission spectra of the modified free dyes (blue dashed line) and the corresponding folate conjugates (red straight line) in a 1% DMSO:H_2O mixture. a) **7c** and N-(2-azidoethyl) dansyl, b) **7d** and fluorescein, c) **7e** and rhodamine 6G and d) **7f** and furazan. The double band structure in the emission of **7c** shown in panel a) is due to a contribution from emission localized in the folate (λ_{em}= 455 nm) in addition to the emission band localized in the dansyl dye (λ_{em}= 552 nm).

In order to illustrate the potential use of these conjugates as markers for cancer cells, the internalization of conjugate **7e** was studied in vitro using cancer (human non-small cell lung cancer, NCI-H460) and non-cancer cell lines (human kidney embryonic cells, HEK 293). Internalization of **7e** is demonstrated in scheme 6 where an overlaid image shows the membrane labelled by WGA-Alexa594 (a typical membrane selective probe) in red and the cytoplasm labelled by **7e** in green. The enhanced uptake of **7e** towards the cancer cell lines



Scheme 6 - Laser scanning confocal images of the HEK 293 (a) and NCI-H460 (b) cells incubated with 5 µM of **7e** for 1 h. The images are an overlaid of the emission of **7e** collected in the 530-580 nm region upon excitation at 476 nm (green color), and the emission of the membrane marker collected in the 670-730 nm region upon excitation at 633 nm (red color). The same incubation and excitation conditions were used in the two images. The corresponding normalized intensity distribution per pixel in the citoplasmatic compartment are displayed in the lower panel (c).

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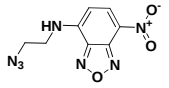
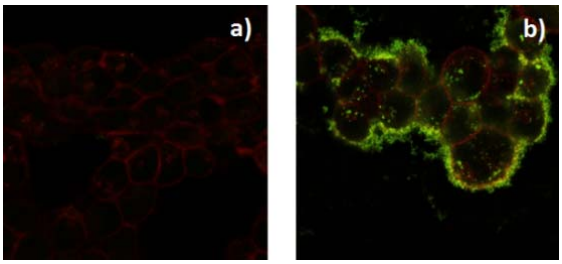


4-(2-azidoethyleneamino)-7-nitrobenzofurazan **8**



Scheme 7 - Laser scanning confocal images of treated NCI-H460 cells for 24 hours: a) cells incubated with 25 μM of **8** and b) cells incubated with 25 μM of **8** together with 12.5 μM of **3**. Images are an overlaid of the emission collected in the 549-591 nm region upon excitation at 488 nm (green color), and the emission of the membrane marker collected in the 700-753 nm region upon excitation at 514 nm (red color). The same incubation and excitation conditions were used in the two images.

is immediately clear by comparing the images of the HEK 293 cells (scheme 6a) with the NCI-H460 cancer cells (scheme 6b). The distribution of pixel counts of a given intensity within the cytoplasmatic compartment of a single cell, shown in the lower panel of Scheme 6, allows for a more quantitative analysis of the increased uptake by the cancer cells. Integration of these distribution curves shows a 5-fold increase in fluorescence inside NCI-H460 cancer cells relatively to HEK 293 non cancer cells, showing that the conjugate **7e** can mark with good selectivity cancer cells over-expressing folate receptors relatively to non-cancer cells. The origin of the two peak structure of the pixel count distribution is not clear but it could be related to the formation of dimmers and higher order aggregates in the cytoplasm due to the high local concentration of the conjugate.

The main advantage of spAAC strategy is the attractive chance of undergoing *in situ* conjugation at living cell, tissue or organisms. To address the possibility of performing *in situ* folate conjugations in living cells, we began by evaluating the efficiency of the click reaction between conjugate **3** and MeO-PEG_{350}-N_3 under physiological conditions. Due to the small solubility of conjugate **3** in water, the reaction was performed in 1% DMSO/water, at 37ºC (112 μM conjugate **3** + 224 μM MeO-PEG_{350}-N_3). After 24 hours, ^{1}H NMR of the isolated folates showed that about 50% of triazole product was formed. Finally, the synthesis of a dye labelled conjugate was attempted in NCI-H460 cells. The comparison between treating the cells with free dye in the presence and absence of conjugate **3** will determine the efficiency of the click reaction in vitro, given that free dye should have the lowest possible bioavailability. For this reason the benzofurazan dye **8** was chosen instead of R6G. NCI-H460 cells were separately incubated during 24 hours with dye **8** (scheme 7a) and with a 2:1 mixture of dye **8** and conjugate **3** (scheme 7b). In the absence of conjugate **3**, no dye **8** is detected inside the cells nor within the cellular membrane. In the presence of conjugate **3** a considerable emission intensity from the **7f** conjugate is observed at the cell membrane (in yellow in scheme 7b), localized with the membrane specific dye (in red in scheme 7b). The trafficking of the dye labelled conjugate to the cell interior exists but is limited. Nevertheless, these results clearly show that the click reaction took place *in situ*.

Conclusions

Herein is described a novel synthetic tool to undergo the functionalization of folic acid and its conjugation into small molecules (including fluorescent dyes), polymers and heterogeneous materials by using efficient non-metal based click chemistry. This methodology presents the following main features:

a) the synthesis of a small folate-cyclooctyne conjugate **3** via an efficient synthetic route displaying over 95% γ-purity and without employing any chromatographic purification;

b) the cycloaddition reaction between conjugate **3** and several fluorescent dyes, polymer and heterogeneous silica bearing azide enables a fast and quantitative synthesis of new folic acid conjugates with high γ-purity;

c) the new folate-rhodamine 6G conjugate **7e** shown higher uptake inside cancer cells lines over-expressing folate receptors (NCI-H460) compared with non-tumoral cell lines;

d) the cycloddition reaction was performed in cellular media leading to an increase of fluorescence inside cancer cells lines over-expressing folate receptors (NCI-H460) due to accumulation of conjugate 7**f**.

The main features presented above allow us to anticipate the potential use of this methodology to easily prepare novel folate conjugates to different specific applications. The fact that the conjugated dyes synthetized herein have decreased quantum yield of fluorescence, will prompt us to undergo optimizations in the spacer unit, in particular by altering the distance between the triazole ring and the cromophores.

Experimental

N-BOC ethylenediamine-folate 5: In a round-bottomed flask was dissolved 640 mg of folic acid (1.34 mmol, 1 eq. dehydrated powder) in 25 ml of DMSO. After the dissolution was complete (about 30 minutes with mild heating), 308 mg (2 eq.) of NHS and 552 mg (2eq.) of DCC were added successively. The reaction mixture was stirred for 16h at room temperature, after which the urea precipitate was filtered off. Then, it was added 0.376 ml (2 eq.) of triethylamine followed by 429 mg (2 eq.) of N-Boc-ethylene diamine dissolved in 5 ml of DMSO. The mixture was again stirred overnight, before was added to a mixture of 20% acetone in diethylether. The thin yellow precipitated was carefully centrifuged and washed four times with acetone and two with diethyl ether and dried under vacuum (658 mg, 73 % yield). The folic acid was conjugated with N-Bocethylenediamine almost exclusively in the terminal carboxylic acid as confirmed by RP-HPLC (injection of sample dissolved in DMSO, 1 ml/min, 0 to 5.5 min 100% water, linear gradient from 5.5min at 100% water to 15.5min at 100% acetonitrile, Gemini 5u 18 110A, 5umx4.6x250mm, Phenomenex column) and ^{1}H NMR. ^{1}H NMR (400 MHz, DMSO) δ 8.64 (s, 1H), 8.04-7.86 (m, 2H), 7.68-7.62 (m, 2H), 7.10-6.82 (m, 3H), 6.65-6.63 (d, 2H), 4.48 (bs, 2H), 4.27 (m, 1H), 3.43 (bs, water) 3.06- 2.88 (m, 4H), 2.28-1.85 (m,4H), 1.35(s, 9H).^{13}C NMR (100 MHz, DMSO) δ 172.38, 166.60, 161.64, 156.07, 154.46, 151.23, 151.19, 149.00, 129.53, 129.32, 128.44, 122.01, 111.72, 78.07, 52.84, 46.43, 46.12, 40.94, 39.27, 32.50, 31.51, 28.68, 10.14.

Ethylene diamine-folate 6: The ethylenediamine-folate conjugate was prepared according with a literature protocol. N-Boc-ethylene diamine folate (300 mg) was dissolved in 2 ml of trifluoroacetic acid and stirred during two hours. The solvent was removed under pressure with aid of dichloromethane and the red-dark residue was dissolved in the minimal amount of dry DMF. The addition of triethylamine resulted in the precipitation of a yellow powder which

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was washed and centrifuged four times with acetone and two times with diethyl ether (207 mg, 83 % yield). 1H NMR (400 MHz, DMSO) δ 8.64 (s, 1H), 8.19-7.96 (m, 1H), 7.66-7.58 (m, 2H), 7.18-6.91 (m, 3H), 6.66-6.63 (m, 2H), 4.47 (bs, 2H), 4.31-4.07 (m, 1H), 3.52 (water), 3.32-3.19 (m), 2.86-2.80 (m, 2H), 2.24-1.93 (m, 4H).

BCN-Ethylene diamine-folate 3: To a solution of ethylenediamine-folate (13 mg, 0.027 mmol) was added diisopropylethyl amine (17 ul, 0.1mmol, 4 eq) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (12 mg, 0.038 mmol, 1.4 eq) and stirred until a clear solution is obtained (about 20 minutes). The reaction mixture was poured into a mixture of 20% acetone in diethylether. The thin yellow precipitated was carefully centrifuged and washed two times with acetone and two times with diethyl ether and dried under vacuum (13mg, 73% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.64 (s, 1H, pterin), 8.14-7.88 (m, 2H), 7.68-7.63 (m, 2H, aromatic), 7.11-6.95 (m, 4H), 6.66-6.64 (m, 2H, aromatic), 4.50 (bs, 2H, benzylic), 4.29 (m, 1H, αH), 4.03-4.01 (m, 2H, -HNCO$_2$C**H**$_2$), 3.36 (water), 3.10-3.02 (m, 4H,O=C-NHC**H**$_2$C**H**$_2$), 2.22-1.92 (m, 10H, C**H**$_2$C**H**$_2$CO$_2$+ CH**H**-CH$_2$-alkyne-CH$_2$-CH**H**+ -C**H**$_2$-alkyne-C**H**$_2$-), 1.52 (m, 2H,CH**H**-CH$_2$-alkyne-CH$_2$-CH**H**), 1.24-1.16 (m, 3H, -HNCO$_2$CH$_2$C**H**), 0.84 (m, 2H, HNCO$_2$CH$_2$CH(C**H**)$_2$).APT NMR (100 MHz, DMSO) δ 172.41, 172.37, 172.26, 166.67, 166.60, 156.91, 154.34, 151.21, 149.07, 148.97, 129.56, 129.40, 128.41, 121.86, 111.65, 99.46, 61.85, 52.60, 46.37, 40.70, 39.13, 32.44, 31.60, 29.03, 21.31, 19.99, 18.07.MS (ESI+) = 660.26 (M+H)$^+$.HRMS (ESI-FIA-TOF) calc. = 660.2894, found = 660.2875.

Conjugate 7a: Method A: BCN-Ethylenediamine-folate (3.5 mg, 0.0051 mmol, 1 eq.) was dissolved in 1 ml of DMSO and added to a flask containing 2 mg of benzyl azide[55] (0,015 mmol, 3 eq.). The reaction was stirred for 2 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 95 % yield (4 mg). ^{1}H NMR (400 MHz, DMSO) δ 11.58, 8.65 (s, 1H), 8.15-8.02 (m, 1H), 7.88 (m, 1H), 7.68-7.66 (m, 2H), 7.37-7.27 (m, 3H), 7.14-6.94 (m, 2H + m, 4H), 6.66-6.64 (m, 2H), 5.54 (s, 2H), 4.50 (bs, 2H), 4.29 4.29 (m, 1H, αH), 4.01-3.97 (m, 2H), 3.36 (water), 3.09-3.00 (m, 6H), 2.86-2.75 (m, 2H), 2.31-1.90 (m, 6H), 1.57-1.46 (m, 2H), 1.08-1.05 (m, 1 H), 0.88-0.76 (m, 2 H).^{13}C NMR (100 MHz, DMSO) δ 172.40, 172.38, 172.25, 166.73, 166.66, 156.93, 156.84, 154.37, 151.21, 148.97, 144.37, 133.50, 129.55, 129.40, 128.41, 128.23, 127.42, 121.87, 111.65, 61.87, 51.11, 46.38, 39.13, 32.45, 25.93, 22.58, 22.09, 21.31, 19.16, 19.04, 17.60. MS (ESI+) = 793.33(M+H) $^+$. HRMS (ESI-FIA-TOF) calc. = 793.3534, found = 793.3522

Method B: Ethylenediamine folate conjugate (10 mg, 0.021 mmol, 1 eq) was dissolved 2ml of DMSO, followed by the addition of diisopropylethyl amine (11ul, 0.063 mmol, 3 eq) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (6.5 mg, 0.022 mmol, 1.05 eq). The reaction mixture was stirred until yellow solution is obtained (about 30 minutes), followed by the addition of benzyl azide (5.6 mg, 0.042 mmol, 2 eq). The reaction was stirred for 2 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 86 % yield (14 mg).

Conjugate 7b: Method A: BCN-Ethylenediamine-folate (3.5 mg, 0.0051 mmol, 1 eq.) was dissolved in 1 ml of DMSO and added to a flask containing 6 mg of MeO-PEG$_{350}$-N$_3$[56] (0,015 mmol, 3 eq.). The reaction was stirred for 5 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 96 % yield (5 mg). ^{1}H NMR (400 MHz, DMSO) δ 8.65 (1H, s), 8.16-8.05 (1H, bm), 7.89 (1H, bm), 7.68- 7.63 (2H, m), 7.13-6.94 (m, 4H), 6.66-6.64 (2H, d), 4.50-4.48 (2H, d), 4.40 (2H, m), 4.29 (1H, bm), 4.03 (2H, bm), 3.74 (2H, bm), 3.50-3.47 (24H), 3.37 (water), 3.24 (3H, s), 3.10- 2.95 (6H, bm), 2.77-2.71 (2H, bm), 2.55-1.92 (6H, bm), 1.55-1.52 (2H, bd), 1.10-1.05 (1H, bm,), 0.92 (2H, bm).^{13}C NMR (75 MHz, DMSO) δ 172.24, 166.68, 156.89, 154.24, 151.20, 149.09, 149.02, 148.96, 143.64, 134.27, 129.57, 129.41, 128.38, 121.84, 111.63, 71.72, 70.22, 70.0, 69.82, 58.49, 55.37, 47.59, 46.33, 25.81, 22.68, 22.42, 21.72, 19.63, 19.08, 17.72.MS (ESI+) = 1025.5 mixture of oligomers with and without Na.

Conjugate 7c: Method B: Ethylenediamine folate conjugate (10 mg, 0.021 mmol, 1 eq) was dissolved 2ml of DMSO, followed by the addition of diisopropylethyl amine (11ul, 0.063 mmol, 3 eq) and (1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (6.5 mg, 0.022mmol, 1.05eq). The reaction mixture was stirred until yellow solution is obtained (about 30 minutes), followed by the addition of N-(2-azidoethyl) dansyl[57] (23 mg, 0.072 mmol, 3.4 eq). The reaction was stirred for 5 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 70 % yield (14 mg).^{1}H NMR (400 MHz, DMSO) δ 8.65 (s, 1H), 8.47-8.45 (d, 1H), 8.22-8.20 (d, 1H), 8.08-8.03 (d, 1H), 7.90-7.88 (bs 1H), 7.68-7.55 (m, 4H), 7.26-7.24 (d, 1H), 7.12-6.92 (m, 4H), 6.66-6.64 (d, 2H), 4.50-4.49 (d, 2H), 4.31-4.24 (m, 3H), 4.01 (m, 2H), 3.36 (H$_2$O), 3.19-3.03 (m, 6H), 2.83-2.68 (m, 8H), 2.34-1.91 (m, 6H), 1.48 (m, 2H), 1.22-1.20 (m, 1H), 0.88 (bs, 2H).^{13}C NMR (100 MHz, DMSO) δ 174.54, 174.44, 172.40, 172.24, 166.69, 156.90, 154.33, 151.83, 151.21, 149.02, 148.98, 143.70, 135.96, 133.83, 130.03, 129.53, 129.41, 129.36, 128.70, 128.40, 128.37, 124.01, 121.88, 119.42, 115.61, 111.66, 65.37, 52.41, 47.57, 46.39, 45.52, 42.82, 40.62, 40.41, 40.20, 39.99, 39.78, 39.57, 39.36, 32.46, 31.12, 25.86, 22.57, 22.37, 21.66, 19.62, 19.18, 17.77.MS (ESI+) = 979.24. HRMS (ESI-FIA-TOF) calc. = 979.3997, found = 979.3967

Conjugate 7d: Method A: BCN-Ethylenediamine-folate (6.9 mg, 0.01 mmol, 1 eq) was dissolved in 2 ml of DMSO and added to a flask containing 10 mg of 3-(1-(2-azidoethyl)thiourea) fluorescein[58] (0,021 mmol, 2 eq). The reaction was stirred for 4 hours before it was poured into a solution of 20 % acetone/diethyl ether. The light orange precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 88 % yield (10.5 mg). ^{1}H NMR (300 MHz, DMSO) δ 11.46 (bs, 1H), 10.16 (bs, 3H), 8.64 (s, 1H), 8.16-7.88 (m, 3H), 7.67 (m, 3 H), 7.20-7.17 (d, 1H), 7.10-6.94 (m, 3H), 6.67-6.55 (m, 8H), 4.49 (m, 4H), 4.28 (m, 1H), 4.00-3.91 (m, 4H) 3.39 (H$_2$O), 3.00 (m, 6H) 2.73 (m, 2H), 2.31-1.91 (m, 6H), 1.55 (m, 2H), 1.23 (s, 1H), 0.93 (bs, 2H).^{13}C NMR (101 MHz, DMSO) δ181.32, 174.43, 174.27, 172.42, 172.25, 168.91, 166.79, 159.94, 157.03, 156.92, 154.20, 152.34, 151.23, 149.17, 148.98, 148.06, 144.00, 141.32, 134.07, 129.49, 128.39, 127.07, 124.64, 121.79, 113.05, 111.66, 110.12, 102.72, 83.50, 65.38, 52.54, 46.36, 43.99, 40.58, 40.37, 40.16, 39.95, 39.74, 39.53, 39.33, 32.47, 30.91, 25.90, 22.61, 22.50, 21.86, 19.76, 19.34, 17.88.MS (ESI+) = 1135.02. HRMS (ESI-FIA-TOF) calc. = 1135.3844, found = 1135.3819

Conjugate 7e: Rhodamine 6G (4-(azidomethyl)phenyl)methyl ether - Rhodamine 6G 4-chloromethyl-1-phenylmethyl ester chloride[59] derivative (30.4 mg, 1 eq.), NaN_3 (6.7 mg, 2 eq.) and NaI (cat.) were dissolved in a mixture of acetone (1 mL) and water (0.5 mL), the resulting mixture was stirred at room temperature during 19.5 hrs. The mixture was extracted with CH_2Cl_2. The organic fraction was dried with Na_2SO_4, filtered and the organics were removed under vacuum. The remaining solid was recrystallized from AcOEt, EtOH and Et_2O to give a greenish pink solid. (8.7 mg, yield= 28%)^{1}H NMR (300 MHz, DMSO) δ (ppm) 8.24 (dd, *J* = 7.6, 1.2 Hz, 1H), 7.83 (dtd, *J* = 19.3, 7.5, 1.4 Hz, 2H), 7.36 (dd, *J* = 7.4, 1.1 Hz, 1H),

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7.12 (d, *J* = 8.1 Hz, 2H), 6.86 (d, *J* = 8.0 Hz, 2H), 6.74 – 6.66 (m, 4H), 4.89 (s, *J* = 10.5 Hz, 2H), 4.77 (s, 4H), 4.42 (s, *J* = 14.5 Hz, 2H), 2.04 (s, 6H), 1.25 (t, *J* = 7.1 Hz, 6H).^{13}C NMR (75 MHz, DMSO) δ (ppm) 164.80, 156.57, 156.42, 155.65, 135.57, 134.51, 133.20, 131.01, 130.34, 129.43, 128.26, 128.17, 125.41, 112.78, 93.56, 66.42, 66.10, 54.97, 53.39, 38.01, 17.74, 13.62. MS (ESI+) = 560.27 HRMS calc for $C_{34}H_{34}ClN_5O_3$: 560.2750, found: 560.2726

Method A: BCN-Ethylenediamine-folate (7 mg, 0.01 mmol, 1 eq) was dissolved in 2 ml of DMSO and added to a flask containing 14 mg of rhodamine 6G (4-(azidomethyl)phenyl)methyl ether (0.024 mmol, 2.4 eq). The reaction was stirred for 4 hours before it was poured into a solution of 20 % acetone/diethyl ether. The purple precipitate was washed four times with acetone and two times with diethyl ether to yield the respective triazole product in 93 % yield (13 mg).^{1}H NMR (400 MHz, DMSO) δ 8.57 (bs, 1H), 8.26-8.23 (d, 1H), 7.88-7.45 (m, 11H), 7.10-6.60 (m, 12H), 5.53 (s, 2H), 4.90 (s, 2H), 4.42-4.22 (m, 3H), 3.98 (m, 2H), 3.46-3.37 (water + C**H**$_2$rhodamine), 3.01 (m, 6H), 2.80 (m, 2H), 2.05-1.92 (m, 12H), 1.51 (m, 2H), 1.29-1.25 (m, 7H), 0.85 (m, 2H).^{13}C NMR (75 MHz, DMSO) δ 172.37, 165.12, 156.93, 156.93, 156.09, 151.25, 150.97, 148.66, 148.22, 144.35, 136.56, 134.72, 133.71, 133.47, 131.42, 130.84, 130.76, 129.74, 128.88, 128.69, 128.49, 128.43, 127.27, 125.79, 113.21, 113.21, 111.77, 111.74, 111.53, 93.94, 66.79, 61.84, 50.80, 46.48, 46.42, 38.65, 38.47, 38.47, 25.99, 25.91, 22.67, 22.12, 21.39, 19.22, 19.15, 19.06, 17.84, 17.84, 17.61, 14.07. MS (ESI+) = 1219.5; HRMS (ESI-FIA-TOF) calc. = 1219.5477, found = 1219.5432.

Conjugate 7f: Method A: BCN-Ethylenediamine-folate (7 mg, 0.01 mmol, 1 eq) was dissolved in 2 ml of DMSO and added to a flask containing 5 mg of N-(2-azidoethyl)-7-nitro benzofurazan **8**[44] (0.02 mmol, 2 eq). The reaction was stirred for 4 hours before it was poured into a solution of 20 % acetone/diethyl ether. The brown precipitate was washed four times with acetone and two times with diethyl ether to yield the respective triazole product in 68 % yield (8 mg).

^{1}H NMR (300 MHz, DMSO) δ 8.64 (bs, 1H), 8.46-8.46 (d, 1H, furazan), 8.01-7.89 (m, 2H), 7.66 (m, 2H), 7.09-6.92 (m, 4H), 6.65-6.62 (m, 2H), 6.30 (d, 1H, furazan) 4.56-4.48 (m, 4H), 4.29 (bs, 1H), 3.96 (m, 3H), 3.02-2.90 (m, 6H), 2.64 (m, 2H), 2.09-1.99 (m, 6H), 1.48 (m, 2H), 1.11-1.07 (m, 3H), 0.8 (m, 2H).

^{13}C NMR (75 MHz, DMSO) δ 172.37, 166.67, 156.94, 156.86, 154.30, 151.20, 149.06, 148.98, 143.93, 138.09, 134.07, 129.54, 129.40, 128.40, 121.86, 121.60, 111.60, 99.83, 65.37, 61.88, 53.47, 46.37, 32.50, 31.15, 25.77, 22.74, 22.44, 21.64, 19.76, 17.74, 15.63.

MS (ESI+, $C_{40}H_{44}N_{16}NaO_{10}$) = 931.33; HRMS (ESI-FIA-TOF, M+H) calc. = 909.3504, found = 909.3488

Conjugate 7g: 1st step – In a Aldrich glass reactor, 3-mercaptopropyl trimethoxysilane (0.36g, 1.8mmol), 6-bromo-1-hexene (0.244g, 1.5 mmol) and 2,2'-azo-bisisobutyronitrile (0.12g, 0.75mmol) were dissolved in 10 ml of chloroform. The reaction was heated for 24h at 80ºC, before the solvent been removed under reduced pressure. The crude was analyzed by ^{1}H NMR, confirming the complete conversion of the alkene, and used in the next step without further purification.[60]

2nd step – The crude thioether was refluxed in 15 ml of dry toluene in presence of flash chromatography grade silica-gel during 24h under Argon atmosphere. After filtrations and washings with dichloromethane, 1.9 g of functionalized silica-gel was isolated accounting for 95 % over two-steps.[60]

3rd step – The bromide functionalized silica-gel (maximum theoretical of 1.5 mmol) was reacted with 650mg of sodium azide (10mmol) in DMF during 72h at 80ºC. The crude solid was filtered and washed thoroughly with water to remove the inorganic by-products salts, yielding 1.4g of a white solid (72% isolated yield).

4th step – Method B: Ethylenediamine folate conjugate (20 mg, 0.041 mmol, 1 eq) was dissolved 4ml of DMSO, followed by the addition of diisopropylethyl amine (22ul, 0.12 mmol, 3 eq) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (13 mg, 0.044 mmol, 1.05eq). The reaction mixture was stirred until yellow solution is obtained (about 30 minutes), followed by the addition of azide-functionalized silica-gel (260mg, maximum theoretical 0.2 mmol of azides). The reaction was stirred for 16h, centrifuged and the light yellow solid (275 mg) was washed thoroughly with acetone and ether. The yellow color was absent from the supernatant indicating fully immobilization. This yellow powder was further eluted with DMSO, and since no leaching of color to the mobile phase was detected it further confirmed the chemical immobilization. Folic acid when physically immobilized in silica-gel is eluted with DMSO. To immobilize physically folic acid in silica-gel, it was dissolved in DMSO, and stirred 30 minutes with silica. After, the DMSO was removed by washing with mixture of 20 % acetone/diethyl ether.

Spectroscopic Methods: The linear absorption spectra were recorded on a Jasco V-660 UV-vis spectrophotometer, and the fluorescence measurements were obtained on a Horiba Jobin Yvon Fluorolog 3-22 Spectrofluorimeter. The spectra were recorded in µmol solutions prepared in spectroscopic grade DMSO. The time-resolved emission fluorescence measurements were performed by time-correlated the single photon timing technique counting (TCSPTC) using the second harmonic of a femtosecond Ti:Sapphire laser (350-470 nm) as an excitation source and a Hamamatsu R2809U-01 MCP-PMT (290-700nm).

Cell staining procedure: Cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotic antimycotic solution (100 units/ml penicillin, 0,1 mg/ml streptomycin and 0,25 mg/ml amphotericin B) and grown in an incubator at 37 ºC and under a 5% CO2 atmosphere.

µ-Slide 8 well plates were coated with poly-L-Lysine for at least 30min and washed before seeding with the cells. After an incubation period of approximately 2 days, cells were treated for 1 hour with 5 µM of the probe to be tested. For conduction of the click reaction in situ, substrates (25 µM of 8 and 12.5 µM of 3) were added to the cells medium and left incubating for a 24 hours period. After treatment, cells were washed with phenol-red free medium and treated with 5 µg/ml of the membrane staining WGA-Alexa594 that was kept 20-30 minutes in contact with the cells. After this staining treatment, cells were washed and fresh phenol-red free medium was added to the wells before imaging.

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Notes and references

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