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Click chemistry on the surface of PLGA-b-PEG polymeric nanoparticles: a novel targetable fluorescent imaging nanocarrier

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Abstract In the quest for biocompatible nanocarriers for biomedical applications, a great deal of effort is put on engineering the nanocomposites surface in order to render them specific to the particular purpose. We developed biocompatible PLGA-b-PEG-based nanoparticles carrying a double functionality (i.e., carboxylic and acetylenic) able to serve as flexible highly selective grafting centers for cancer diagnosis and treatment. As a proof of concept, the nanocarrier was successfully functionalized with a tailored fluorescent molecule by means of click chemistry and with a targeting agent specific for glioblastoma multiforme via amidic bond formation.

Keywords Click · Fluorescein · Functionalized polymers · Nanochemistry · Glioblastoma

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A. Pucci · E. Locatelli · V. Molinari ·
M. Comes Franchini (✉)
Dipartimento di Chimica Industriale “Toso Montanari”, University of Bologna, Viale del Risorgimento 4, 40136 Bologna, Italy
e-mail: mauro.comesfranchini@unibo.it

J. Ponti · C. Uboldi
Nanobiosciences Unit, Institute for Health and Consumer Protection, Joint Research Centre, Via E. Fermi 2749, 21027 Ispra, VA, Italy

Introduction

The addition of functional groups to the surface of nanoparticles (NPs) is usually highly required to enable the bioconjugation, and therefore, to obtain multifunctional nanobiomaterials. Generally, linkers with terminal amino, thiol, or carboxylic groups are preferred (Hermanson 2008). However, these functional groups are quite common and abundant in the biomolecules; thus, even though often used, in some cases, they could compromise selective conjugation. Moreover, charged species (NH_2 or COOH) give rise to electrostatic (unspecific) interactions that could lead to particles aggregation.

Nevertheless, the multiple presence of functional groups on nanocarriers would certainly be an important item for the synthesis of biohybrid polymeric nanoparticles (PNPs) (Metallo et al. 2003) and the combination of tracking agents, synthetic polymers, and biomolecules into new classes of nanomaterials has indeed gained much interest in recent years and has now become an important field in diagnostics.

Among the most important synthetic polymers, the poly(lactic-co-glycolic acid)-block-poly(ethyleneglycol) (PLGA-*b*-PEG) deserves a special mention (Gu et al. 2008). Our interest is (Locatelli and Comes Franchini 2012; Gentili et al. 2009) in the PLGA-*b*-PEG–COOH due to special properties, such as amphiphilicity and its simple synthesis. It is indeed a unique biomaterial: it possess both the biodegradability and biocompatibility of PLGA polymer and the stealth

behavior of PEG. Due to its well-distinct lipophilic (PLGA) and hydrophilic (PEG) portions, it can form micelles displaying internally the hydrophobic PLGA part and externally the hydrophilic PEG, which forms a stabilizing external shell. These unique physico-chemical characteristics and behavior make the micelles extremely versatile and able to easy entrap, in the inner core constituted of PLGA chains, lipophilic molecules such as drugs or smaller nano-structures. The residual functional groups on the outer shell linked to the PEG can be further be elaborated from the synthetic point of view.

Taking into consideration, the above mentioned problem related to the common functional groups, there is a well-established combination of functional groups that emerged in the last years. Huisgen 1,3-dipolar cycloaddition (1,3-DC), involves the reaction of an azido group to an alkyne group and provide fast access to an enormous variety of robust and stable heterocycles called triazoles (Huisgen 1984). This so called "click-chemistry" is therefore an attractive reaction because these two organic functional groups are hardly present in proteins and oligomers. The application of click-chemistry in bioconjugation reactions works for molecularly dissolved species, but can also be used to functionalize the nanostructured materials, giving thus an easy entry to hybrid multi-functional nanobiomaterials (Opsteen et al. 2008). Moreover, the catalytic effect of Cu(I) ions of this cycloaddition, discovered by Meldal (Tornøe et al. 2002) and Sharpless (Rostovtsev et al. 2002), gave a great stimulus to the application of this reaction (Moses and Moorhouse 2007). Notably, in terms of biological applications, as the bioconjugation, the metal-catalyzed click 1,3-DC proceeds in high yields and occurs at room temperature in aqueous solution at neutral pH.

Therefore, a polymeric nanocarrier based on PLGA-*b*-PEG carrying a double functionality of such kinds at its hydrophilic surface, and so enabling easy docking of diverse organic bioactive species and tracking agents, would represents a perfect support for theranostic purposes or drug delivery.

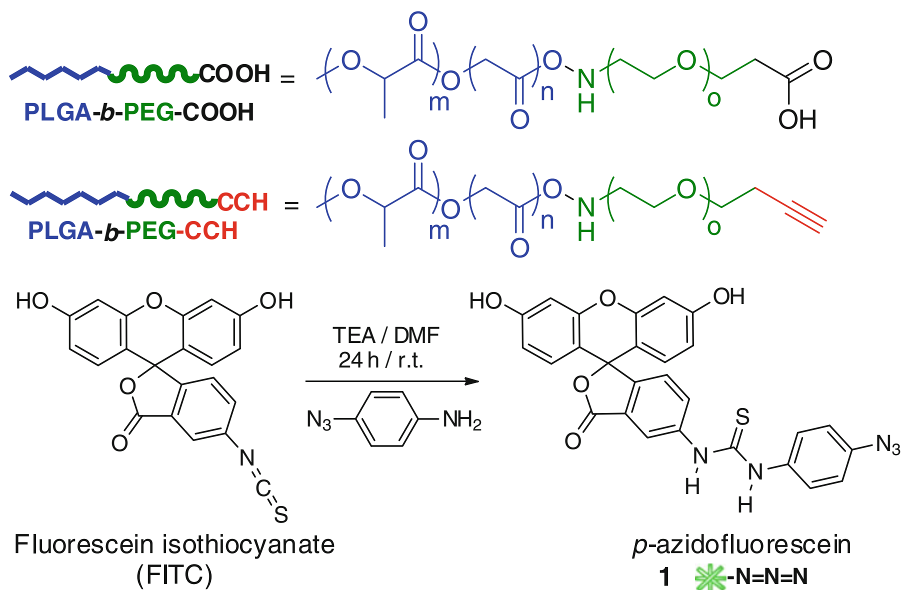
Concerning the tracking, fluorescence imaging is one of the most powerful technique currently available for continuous observation of dynamic intracellular processes in living cells (Johnson 1998; Agarwal et al. 2008; Urano et al. 2005). Fluorescein was first developed in the nineteenth century (Weber and Teale 1958), and has become widely known as a highly fluorescent molecule that emits longer wavelength light upon excitation at around 500 nm in aqueous media. Bioconjugation on polymeric micelles with fluorescent moieties is still an important challenge (Wang et al. 2009), therefore, the functionalization of the commercially available fluorescein isothiocyanate (FITC) in order to link the fluorescent part onto the surface of PNPs would be an important synthetic goal.

We report an innovative biohybrid nanomaterial based on the copolymer PLGA-*b*-PEG, having multiple outer shell decoration for selective double functionalization. In details, one functional group is an acetylenic group to develop a click-reaction with a synthetic azidofluorescein. The second functional group, the carboxylic group, is used to conjugate the Chlorotoxin (Cltx), a specific peptide for in vitro targeting of the human primary brain tumor glioblastoma U87MG cell line.

Results and discussion

While the PLAG-*b*-PEG–COOH has been previously fabricated (Cheng et al. 2007) and it was reproduced in this work following a similar methodology, the copolymer ending with an acetylenic (CCH) group PLGA-*b*-PEG–CCH was synthesized for the first time by readapting the same procedure and replacing the NH_2–PEG–COOH reagent by its acetylenic commercial counterpart NH_2–PEG–CCH. The two-steps protocol is straightforward and leads to a clean and stable co-polymer in 85 % yield (Scheme 1). A derivative of fluorescein, which was synthesized for the first time by a common nucleophilic addition reaction between the FITC and the amino group of a *p*-azidoaniline, was chosen as a fluorescent dye. As reported in Scheme 1, the reaction was carried out in *N*,*N*-dimethylformamide (DMF) with triethylamine (TEA) giving 1-(4-azidophenyl)-3-(3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthen]-5-yl)thiourea **1** in 90 % yield. A similar approach to obtain azido-terminal fluorescein was already pursued exploiting more expensive and hazardous linkers (Lin et al. 2006). The substituted fluorescein **1** carries now a pendant azido group which can react with an acetylenic moiety via the copper-catalyzed cycloaddition (CuAAC) reaction.

Scheme 1 Structures of PLGA-*b*-PEG-based polymers and synthesis scheme of 1-(4-azidophenyl)-3-(3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthen]-5-yl)thiourea **1**

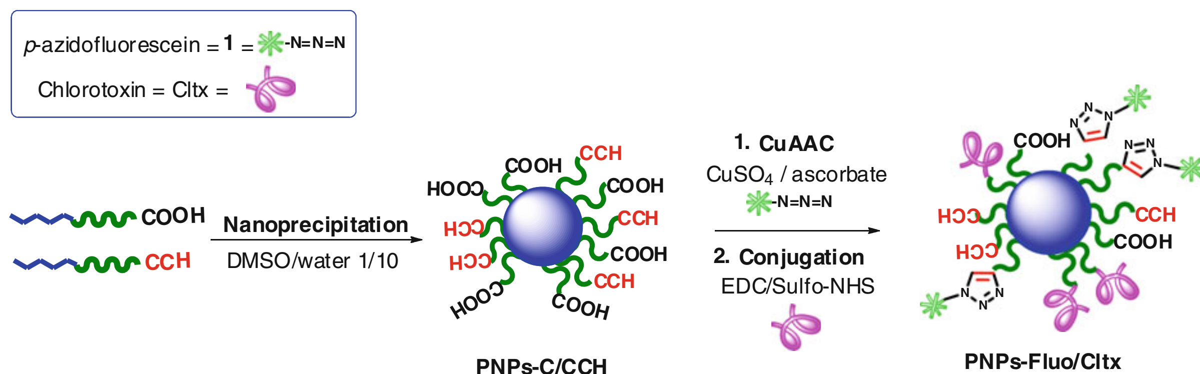


The PNPs fabrication was achieved through the nanoprecipitation technique (Comes Franchini et al. 2010) by solubilizing in DMSO a 50:50 blend of the two copolymers PLGA-*b*-PEG–COOH and PLGA-*b*-PEG–CCH, and subsequently mixing it with water under vigorous stirring and maintaining the water/organic ratio 10:1 with constant removal of the solution (Scheme 2). The as-fabricated nanocarrier PNPs–C/CCH presents a hydrodynamic size of 136.4 ± 0.9 nm with a rather small polydispersity ($\mathrm{PDI} = 0.067 \pm 0.018$). The bifunctionalized surface of the PNPs leads to a multimodal distribution of the ζ-potential with peaks falling in a voltage range lower than −50 mV, indicating very high stability of the NPs (see DLS results in the supporting information). The DLS analyses were repeated over time up to 4 months showing no major changes. Thus, we can assume that the NPs are stable for several months if stored at 4 °C. Next, we focused our attention on the tailoring of the surface functionalities in order to exploit the fertile docking centers. Indeed, the polymeric nanocarriers PNPs–C/CCH represent a perfect support enabling easy docking of diverse organic bioactive species and tracking agents. In order to prove the applicability, the nanocarrier was functionalized with, on one hand, the as-synthesized fluorescent dye **1** and, on the other hand, the peptide Cltx.

The click-reaction ensures high specificity as well as processability in water (Rostovtsev et al. 2002). The water solution of PNPs–C/CCH was mixed with **1** in the presence of a catalytic amount of $CuSO_4$ and sodium ascorbate (Scheme 2). Upon reacting overnight at room temperature, the fluorescent moiety was linked, creating a triazole ring, onto the surface giving PNPs–C/Fluo, and leaving the COOH group still available. Using the typical 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemistry, we then chemically linked the commercially available Cltx to the outer surface (COOH, ratio Cltx:COOH 8.5:1) of the PNPs–C/Fluo. The reaction was carried out overnight at room temperature, and after purification we obtained PNPs-Fluo/Cltx. It is worth mentioning that the two organic molecules attached on the surface sparingly decrease the stability of the particle leading to a hydrodynamic size of 180.3 ± 3.1 nm with a PDI of 0.146 ± 0.014 and a ζ-potential of −38.1 mV at nearly neutral pH.

The sequence of the reaction proved not to be indifferent. Trials were also carried out conjugating the Cltx onto the nanocarrier surface as a first step, prior to the cycloaddition reaction. The as-fabricated nanocarriers presented bigger size as well as higher polydispersity at the DLS (data not shown). Evidently, once the 36-amino acids peptide is linked on the surface, it constitutes an obstacle to the copper-catalyzed click reaction.

The luminescence properties of the PNPs-Fluo/Cltx together with the one of azidofluorescein and of the



Scheme 2 Schematic of the PNPs-Fluo/Cltx fabrication

bare PNPs were monitored using a spectrofluorometer. The excitation and emission spectra are depicted in Fig. 1a, b, respectively. The excitation of the azido-terminal fluorescein occurs at 460 nm and it undergoes a red shift to 491 upon linkage onto the particle surface. The green intense emission falls at 522 and at 516 nm for the *p*-azidofluorescein and the PNPs-Fluo/Cltx, respectively. As expected, the polymeric NPs absorb in the near UV (309 nm), and consequently do not emit when excited in the blue spectral range.

We decided to link the Cltx, a peptide that specifically binds to metalloproteinase 2 (MMP-2), a receptor over-expressed by brain cancer (glioblastoma cells) due to our interest in this research (Comes Franchini et al. 2010; Locatelli et al. 2012), therefore, U87MG cells (a stabilized cell line derived from human glioma) were exposed to 0.88 μg/mL of PNPs-Fluo/Cltx. This testing concentration is no toxic, as previously observed (Locatelli et al. 2012). In fact, U87MG cells were exposed to 50 μg/mL of PNPs-Cltx for 72 h, and by trypan blue exclusion dye test, we observed cell viability of 84 % of the unexposed cells (negative control) (Locatelli et al. 2012).

As shown in Fig. 2, PNPs-Fluo/Cltx (in green, in Fig. 2b, e) were internalized by U87MG glioblastoma–astrocytoma cells after both 48 (Fig. 2a–c) and 72 h (Fig. 2d–f) of exposure. Micelles were mainly localized in the cytoplasm and we excluded their internalization in the nuclei analysing each image of the slices obtained by Z-stack (ApoTome slide module, Carl Zeiss, Germany). At the concentrations tested, they did not alter the cell morphology (Fig. 2c–f) showing, also considering the toxicological results, biocompatibility of the system. Our results suggest that the PNPs-Fluo/Cltx, thanks to the presence of both a fluorescent dye and to the specificity of Cltx for cancer cells, could be potentially used as targeting agent for diagnostic and therapy.

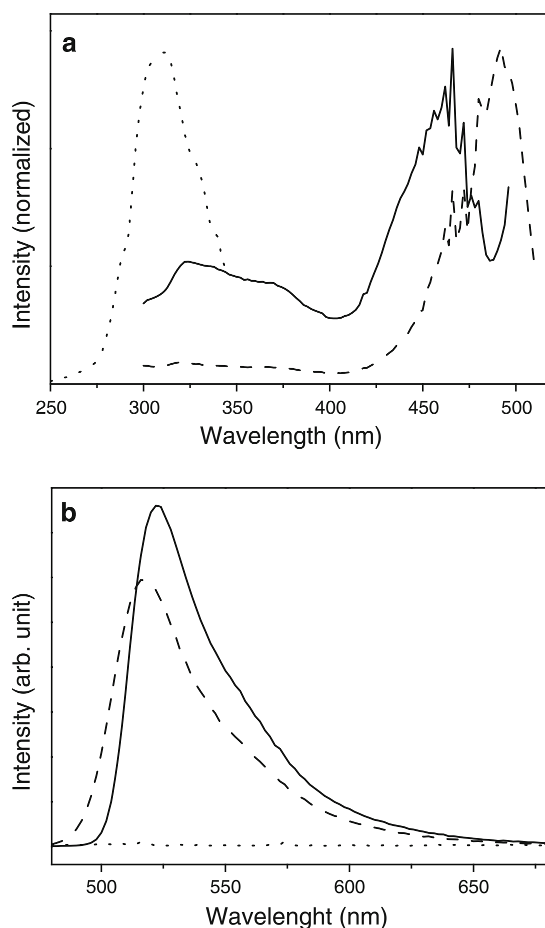


Fig. 1 Room temperature excitation (**a**) and emission (**b**) spectra of the *p*-azidofluorescein (*solid line*), PNPs-Fluo/Cltx (*dashed line*), and the PNPs–C/CCH (*dotted line*)

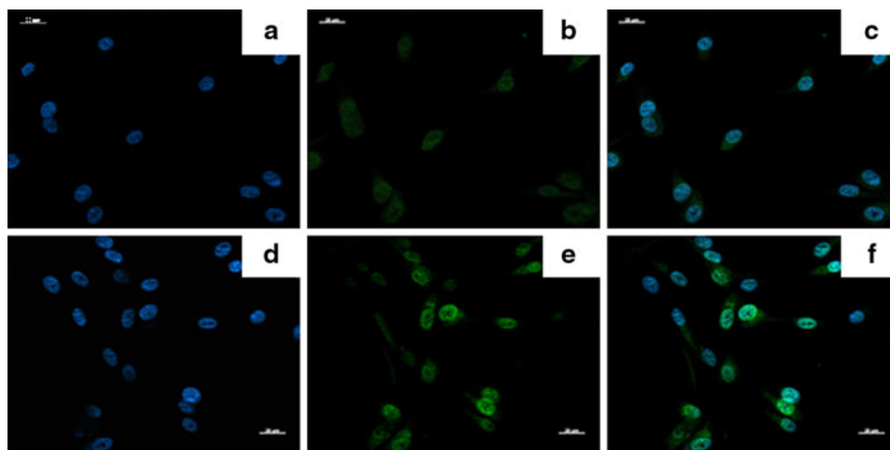


Fig. 2 Intracellular localization of PNPs-Fluo/Cltx in U87MG cells. After 48 h (**a**–**c**) and 72 h (**d**–**f**) exposure, PNPs-Fluo/Cltx micelles were observed located in the cytoplasm of U87MG cells. **a**, **d** in *blue* are nuclei stained with Hoechst 33342; **b**, **e** in *green* are PNPs-Fluo/Cltx micelles; and **c**, **f** merge. Size *bar*: 20 µm. (Color figure online)

Conclusion

In conclusion, we have developed the synthesis of a simple polymeric NPs based on PLGA-*b*-PEG having two functional groups on the outer shell. This nanocarrier bears an acetylenic group on which we have linked a fluorescent tag using a click reaction and, moreover, a carboxylic group, that is able to conjugate peptides and proteins through simple amidation chemistry. As already mentioned, the click chemistry is becoming a major pathway among the biological conjugations and many dyes or bioactive molecules carrying an azido pendant are already commercially available. On the other hand, engineering the nanocarrier surface by modifying the pristine polymers instead of venturing on later modification seems to be a preferable approach which can be further expanded to a large variety of terminal groups. Accordingly, this novel targetable nanocarrier has been tested in the in vitro tracking of a glioma cell line, and is therefore currently being investigated in our laboratories for active drug delivery both in vitro and in vivo against glioblastoma.

Experimental section

Synthesis of PNPs–C/CCH

PLGA-*b*-PEG–COOH (10 kDa, 25 mg, 0.0025 mmol) and PLGA-*b*-PEG–CCH (10 kDa, 25 mg, 0.0025 mmol) were solubilized in 5 mL of DMSO. The organic phase was mixed to 50 mL of ultrapure water under vigorous stirring, maintaining water/organic ratio 10/1 with a constant removal of the solution. The mixture was kept for 30 min under vigorous stirring. The solution was concentrated to a volume of 10 mL using a tangential flow filter (Pellicon XL filter device, Biomax membrane with 500.000 NMWL, Millipore, USA) following by filtration using a syringe filters Sterivex^{TM}-GP of polyethersulfone (0.22 µm, Millipore, USA). Hydrodynamic size = 136.4 ± 0.9 nm, PDI = 0.067 ± 0.018, and ζ-potential = trimodal, far above the stability threshold.

Synthesis of PNPs–C/Fluo and PNPs-Fluo/Cltx

A water solution of PNPs–C/CCH (2 mL, –COOH and –CCH 1 µmol) was mixed with the *p*-azidofluorescein **1** (40 mg, 0.076 mmol) together with copper sulfate hydrate (0.7 mg, 0.0028 mmol) and (+)-sodium L-ascorbate (0.3 mg, 0.0015 mmol). The mixture was allowed to stir overnight. The resulting particles were washed with ultrapure water (up. water) using a centrifugal (3000 rpm, 15 min) filter devices (Amicon Ultra, Ultracel membrane with 100.000 NMWL, Millipore, USA) till the washing water resulted in a colorless fluid. Finally, the particles were redispersed in 5 mL of up. water.

To this dispersion, a water solution containing *N*-hydroxysulfosuccinimide (0.1 mg, 0.46 µmol) was added together with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 2 mg, 0.012 mmol) that was

pre-acidified with 0.1 M HCl solution to neutral pH ($\sim$7). The solution was shacked for 30 min to permit the activation. Next, the Cltx (0.5 mL of a 0.017 M solution, 0.0085 mmol) was added to the reaction mixture and it was allowed to shake overnight. The resulting particles were washed with up. water using a centrifugal (3000 rpm, 15 min) filter devices (Amicon Ultra, Ultracel membrane with 100.000 NMWL, Millipore, USA). Finally, the particles were filtered through a Sterivex^{TM}-GP of polyethersulfone (0.22 μm, Millipore, USA). Hydrodynamic size = 180.3 ± 3.1 nm, PDI = 0.146 ± 0.014, ζ-potential = −38.1 mV, and pH = 7.04. Polymer concentration obtained by weighing a dried aliquot of the final solution = 1.226 mg/mL.

Intracellular localization of PNPs-Fluo/Cltx

To perform microscopy studies, cells were seeded onto 4-chamber polystyrene vessel tissue culture-treated glass slides (BD Falcon, Italy) in 1 mL of complete cell culture medium and cultured under standard cells culture conditions (37 °C, 5 % CO_2, 95 % humidity). After 24 h, cells were exposed to 0.88 μg/mL of PNPs-Fluo/Cltx for 48 h and 72 h; then fixed with 4 % (v/v) formaldehyde (Sigma Aldrich, Italy) solution in PBS and stained using Hoechst-33342 (Invitrogen, USA) for nuclei. Images were acquired using an Axiovert 200 M inverted microscope (Carl Zeiss, Germany) equipped with ApoTome slide module and AxioVision 4.8 software (Carl Zeiss, Germany), using 40×/1.0 objective lens. Images were acquired using a black and white AxioCam MRm (Carl Zeiss, Germany) and pseudo-colors were applied after image acquisition.

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