Available online at www.sciencedirect.com

ScienceDirect

Procedia Engineering 87 (2014) 751 – 754

Procedia Engineering

www.elsevier.com/locate/procedia

EUROSENSORS 2014, the XXVIII edition of the conference series

Complex nanostructures based on oligonucleotide optical switches and nanoparticles for intracellular mRNA sensing and silencing

B. Adinolfi[a], S. Carpi[b], A. Giannetti[a], P. Nieri[b], M. Pellegrino[3], G. Sotgiu[d], S. Tombelli[a], C. Trono[a], G. Varchi[d], F. Baldini[a]*

[a]*Istituto di Fisica Applicata "Nello Carrara", Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, 50019 Sesto Fiorentino (FI), Italy*
[b]*Dipartimento di Farmacia, Università di Pisa, Via Bonanno, 6, 56126 Pisa (PI), Italy*
[c]*Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie in Medicina e Chirurgia, Università di Pisa, Via Savi 1, 56126 Pisa (PI), Italy*
[d]*Istituto per la Sintesi Organica e la Fotoreattività, Consiglio Nazionale delle Ricerche, Via P. Gobetti 101, 40129 Bologna (BO), Italy*

Abstract

The design, implementation and characterization of complex nanostructures for intracellular mRNA monitoring constituted by a molecular beacon (MB), covalently immobilized onto polymethylmethacrylate (PMMA) fluorescent nanoparticles (NPs) are described. The MB molecule, adsorbed onto the nanoparticle for intracellular delivery, is capable to turn on the fluorescence emission of its fluorescent label, upon the interaction with a well-defined target mRNA. The mRNA specific for survivin has been chosen as target. A MB for survivin mRNA was characterized in vitro to verify its functionality. The functionality and specificity of the MB were also examined in living cells by transfection with a classical lipid agent, Lipofectamine. In addition, bear and MB-coated PMMA NPs were tested with adenocarcinomic human alveolar basal epithelial cells (A549) in terms of cell vitality and internalization.

Peer-review under responsibility of the scientific committee of Eurosensors 2014

Keywords: Molecular beacon; mRNA; survivin; PMMA nanoparticle

1. Introduction

The concept of antisense technology as drugs is immediate: the use of a complementary sequence to the mRNA,

* Corresponding author. Tel.: +39 0555226323 fax: +39 0555226400.
E-mail address: f.baldini@ifac.cnr.it

Peer-review under responsibility of the scientific committee of Eurosensors 2014
doi:10.1016/j.proeng.2014.11.645

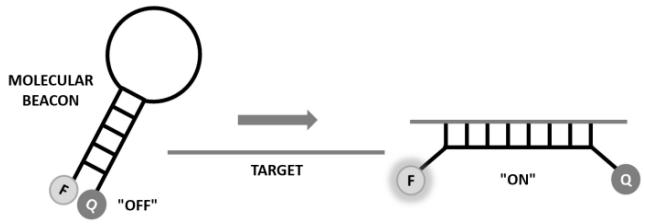

Fig. 1. Structure of a molecular beacon (MB) and its interaction with the specific target.

following the hybridization rules of Watson and Crick, can inhibit its expression then induce a stop in the transfer of the information from the DNA to the protein synthesis [1, 2]. Recently, the antisense technology has been combined with nanosensing feature, potentially able to provide real time monitoring of intracellular events [3], merging the antisense drug property with the optical switch capability. Oligonucleotide optical switches are suitable molecules capable of turning on or modifying their light emission on molecular interaction with well-defined molecular targets. The use of an antisense oligonucleotide optical switch, able to generate a fluorescent signal when it hybridizes with the target mRNA, may represent an innovative strategy that conjugates the ability of sensing specific mRNA with the pharmacological silencing activity preventing the overexpression of proteins associated to pathologic conditions. Among these types of antisense oligonucleotide, molecular beacons (MBs) are very promising tools to optically monitor their interaction with the target molecule. They are formed by oligonucleotidic sequences labeled at the two side ends, with a fluorophore F_A on one side and either a quencher Q or a second fluorophore F_B on the other side, with the sequence properly designed so as to form a hairpin structure (stem-loop) when the complementary sequence is not present (Figure 1). In this condition, in the absence of the complementary target, the two labels are only a few angstroms separated and the fluorophore F_A, if excited when the MB structure is closed, does not exhibit fluorescence but transfer the absorbed energy to the second label (fluorescence resonance energy transfer). No emitted light - if the second label is a quencher - or fluorescence at a longer wavelength than that one of the F_A fluorescence - if the second label is a second fluorophore - is observed. In the presence of the target, the MB opens and the fluorophore F_A is no more so close to the second label as to consent the energy transfer to the second label, and fluorescence at its emission wavelength is observed.

The use of carriers for the intracellular drug delivery or for the transportation of drugs in target cells, difficult to get in normal conditions, is a well-known strategy and the use of traditional delivery structures, more or less complex, such as liposomes, viruses antibodies, micelles, is the applied strategy [4]. With the advent of new nano-sized materials, fascinating possibilities in the transport within the cells are appeared. The development of efficient methodology for the chemical modification has led to the preparation of functionalized nanoparticles [5, 6] on which a large variety of active molecules can be immobilized (e.g. peptides, proteins, oligonucleotidic chains and other therapeutic agents). An important aspect is the fact that the modified nanostructures have been shown to be able to cross the cellular membranes under well-controlled conditions with suitable mechanisms [7].

In the present paper, we describe the design, implementation and characterization of complex nanostructures for intracellular survivin mRNA monitoring constituted by a molecular beacon, adsorbed on polymethylmethacrylate (PMMA) fluorescent nanoparticles (NPs). The mRNA specific for survivin has been chosen as target, being survivin a multifunctional protein that plays a role in cancer development and progression [8] and highly overexpressed in cancer cells.

2. Materials and methods

PMMA nanoparticles were synthesized at ISOF-CNR by emulsion polymerization and they were constituted by a core of PMMA surrounded by a shell bearing cationic quaternary ammonium (498 μmol/g) and $–NH_2$ (208 μmol/g) groups. The same nanoparticles were also functionalized, during the synthesis, with fluoresceine.

The survivin molecular beacon was purchased from IBA (Gottingen, Germany) with the following sequence [9]: 5’-(ATTO647N)CGACGGAGAAAGGGCTGCCACGXCG(BBQ)-3’ X=C6-dT Thio. The underlined sequence

represents the specific sequence for survivin mRNA. Atto 647N (λ_{abs} 644 nm, λ_{em} 669 nm) and Blackberry Quencher 650 (λ_{max} ~ 650 nm, useful absorbance between 550 and 750 nm) were used as fluorophore/quencher pair. The MB complementary oligonucleotide (target 5'-CCCCTGCCTGGCAGCCCTTTCTCAAGGACC-3') and a non-specific sequence (random, 5'-ATCGGTGCGCTTGTCG-3') were purchased from MWG Biotech (Milan, Italy). RPMI1640 was purchased from Invitrogen.

Fluorescence measurements were carried out in a cuvette using, for excitation, a PicoQuant (LDH-P-C-635B) laser diode emitting at 635 nm filtered with a bandpass interference filter (ThorLabs FL635-10) at 635. An optical fibre (diameter 200 μm) oriented at 90° with respect to the direction of the excitation beam and with a GRIN lens on its tip was used to collect the fluorescent collimated signal and to send it to an Andor Shamrock 303 spectrograph. Cell images were obtained by a confocal microscope Biorad RadiancePlus and the Z stack images were analysed with ImageJ software with MEDNUC or 3D Volume Viewer plugins.

3. Results and discussions

3.1. Molecular beacon in solution

The molecular beacon targeting survivin mRNA was firstly characterized in solution, by evaluating its capability of binding the complementary sequence. A solution of 100 nm of MB in buffer (Tris 10 mM, $MgCl_2$ 10 mM, pH 8) was exposed to different concentrations of the target from 0 to 1000 nM. Fluorescence measurements were conducted after 1, 3 and 5 h of incubation of the MB with the different solutions of target and very little variability was observed.

The calibration curves in Figure 2a shows the correct MB working with the increase of the fluorescence following an increase in the target concentration. Moreover, the MBs resulted to be specific for the target since the signal in presence of the non-specific sequence (100 nM) was equal to the one with only buffer.

The functionality and specificity of the MB were also examined in living cells by experiments performed on the human melanoma A375 cell line and using human monocytes as negative control. The MB was firstly transfected by the classical lipid agent, lipofectamine. Our findings demonstrated a fluorescence increase in the cytoplasm 1 h after beginning the transfection without evidence of fluorescence signals in the extracellular environment (Figure 2b). Moreover, no fluorescence was observed in transfected cells that did not express survivin (monocytes).

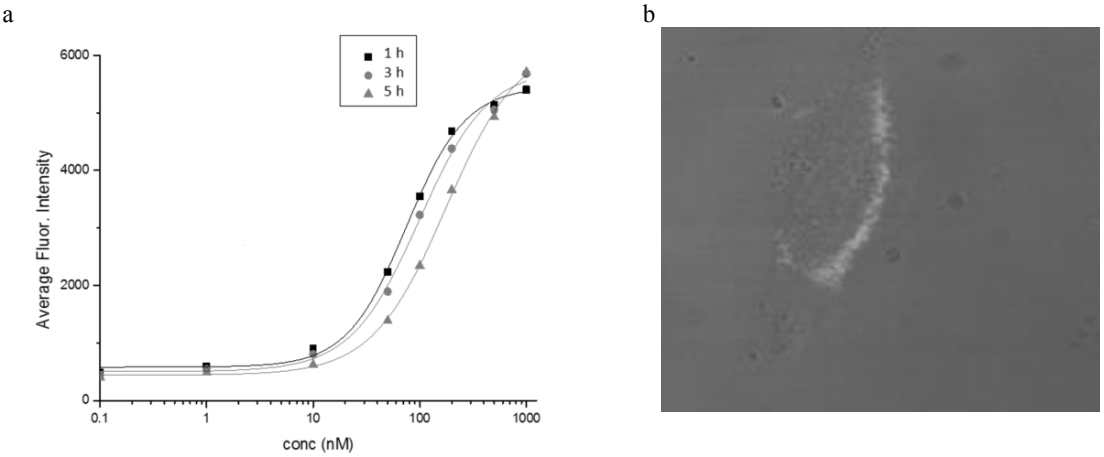

Fig. 2. (a) calibration plot for the molecular beacon 100 nM with increasing concentrations (1-1000 nM) of the target (λ_{ex} 635 nm). The different curves are related to different incubation times; (b) A375 cells treated for 1 h with 100 nM MB-lipofectamine.

3.2. Molecular beacon adsorbed onto nanoparticles in cells

The effects of different concentrations of PMMA nanoparticles on the adenocarcinomic human alveolar basal epithelial cells (A549) viability were analyzed by WST-1 assay, a colorimetric test that evaluates the mitochondrial enzymatic activity of the cells as index of cell viability. The results, expressed as % of cell viability vs an untreated control, showed a not toxic profile of activity of the PMMA nanoparticles up to 25 μg/mL (data not shown). On the basis of these results and in order to guarantee the visualization of suitable fluorescent signals, the concentration 10 μg/mL was chosen for the localization studies realized by confocal microscopy. In this phase, the experimental conditions have been optimized considering to use RPMI1640 supplemented with 10% FBS and a time of exposure of 1 h and 30 minutes which was the minimum time of treatment to which it was possible to state with certainty the intracellular localization of the nanoparticles.

MB-adsorbed onto NPs was also tested on the A549 cells in terms of internalization. These experiments provided a clear evidence of the subcellular distribution of PMMA NPs in living cells as well as of their ability to promote the MB internalization (Figure 4).

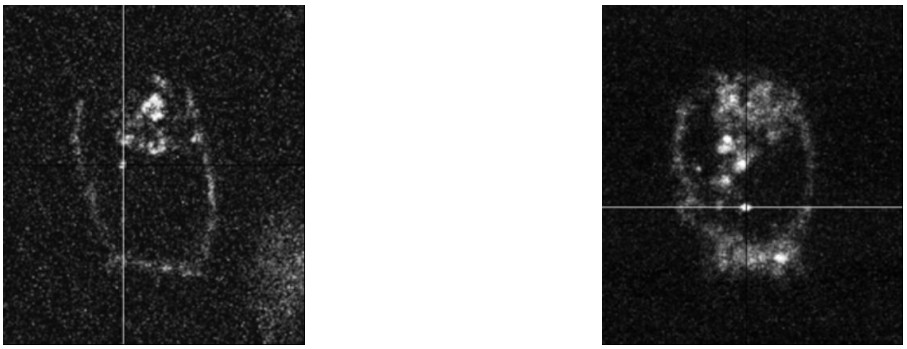

Fig. 4. Fluorescence images by confocal microscope of an A549 cell treated with the MB (100 nM) adsorbed onto PMMA NPs (10 μg/mL) for 1 h and 30 minutes. Left: image of the cell with λ_{ex} = 488 nm (NPs); right: image of the cell with λ_{ex} = 635 nm (MB).

4. Conclusion

We have shown that oligonucleotide optical switches, together with NPs, can play a fundamental role in achieving quantitative information on intracellular events. The conducted analytical characterization has demonstrated that they can be used not only as simple on-off elements but also as real sensors.

Acknowledgements

This work was supported by the regional project NANOCELL (PAR FAS REGIONE TOSCANA Linea 1.1.a.3) and by the national flagship project NANOMAX.

References

[1] N. Dias, C.A. Stein, Antisense Oligonucleotides: Basic Concepts and Mechanisms, Mol. Cancer Ther. 1 (2002) 347-355.
[2] Y. Zhang, X. Yong Lei, Effect of bcl-2 antisense oligodeoxynucleotides on drug sensitivity of leukemic cells, Hemat. J. 4 (2003) 187-197.
[3] A. Giannetti, S. Tombelli, F. Baldini, Oligonucleotide optical switches for intracellular sensing, Anal. Bioanal. Chem. 405 (2013) 6181–6196.
[4] N.G. Portney, M. Ozkan, Nano-oncology: drug delivery, imaging and sensing, Anal. Bioanal. Chem. 384 (2006) 620-630.
[5] S. Banerjee, T. Hemraj-Benny, S.S. Wong, Covalent surface chemistry of single-walled carbon nanotubes, Adv. Materials, 17 (2004) 17-29.
[6] G. Han, P. Ghosh, V.M. Rotello, Functionalized gold nanoparticles for drug delivery, Nanomedicine, 2 (2007) 113-123.
[7] T.M. Fahmy, P.M. Fong, A. Goyal, W.M. Saltzman, Targeted for drug delivery, Materials Today 8 (2005) 18-26.
[8] J.A. McKenzie and D. Grossman, Role of the Apoptotic and Mitotic Regulator Survivin in Melanoma, Anticancer Res. 32 (2012), 397-404.
[9] N. Nitin, P.J. Santangelo, G. Kim, S. Nie, G. Bao, Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells, Nucl. Acids Res. 32(2004), e58.