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Long period fiber grating nano-optrode for cancer biomarker detection

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ABSTRACT

We report an innovative fiber optic nano-optrode based on Long Period Gratings (LPGs) working in reflection mode for the detection of human Thyroglobulin (TG), a protein marker of differentiated thyroid cancer.

The reflection-type LPG (RT-LPG) biosensor, coated with a single layer of atactic polystyrene (aPS) onto which a specific, high affinity anti-Tg antibody was adsorbed, allowed the label-free detection of Tg in the needle washouts of fine-needle aspiration biopsies, at concentrations useful for pre- and post-operative assessment of the biomarker levels.

Analyte recognition and capture were confirmed with a parallel on fiber ELISA-like assay using, in pilot tests, the biotinylated protein and HRP-labeled streptavidin for its detection. Dose-dependent experiments showed that the detection is linearly dependent on concentration within the range between 0 and 4 ng/mL, while antibody saturation occurs for higher protein levels. The system is characterized by a very high sensitivity and specificity allowing the *ex-vivo* detection of sub ng/ml concentrations of human Tg from needle washouts of fine-needle aspiration biopsies of thyroid nodule from different patients.

1. Introduction

The ever increasing incidence of cancer diseases is imposing the development of highly sensitive and effective tools for the real-time detection of associated biomarkers for early diagnosis. This is particularly needed for the diagnosis of papillary thyroid cancer, whose incidence has dramatically increased over the past few years in the United States and is predicted to increase in the next years, recording a greater frequency in the female population (Weir et al., 2015). Papillary thyroid cancer is the most common malignancy of the thyroid. Although it has favorable long-term survival in most cases, an early stage diagnosis is fundamental to start therapies that reduce mortality and lessen the associated endocrine disorders. The frequency of lymph nodes involvement is 27–46% at initial diagnosis and the recurrence rate is 3–30% during post-operative follow-up. Distinguishing lymph nodes metastasis from benign reactive lymphadenitis is therefore critical to rank the malignancy risks in patients with papillary thyroid cancer (Moon et al., 2013). Thyroglobulin (Tg) is a 660 kDa dimeric protein produced by and used entirely within the thyroid gland to produce the thyroid hormones thyroxine and triiodothyronine. Serum Tg levels are elevated in patients with goiter and in several other clinical conditions, and to date, the measurement of Tg is the mainstay in the post-surgical follow-up of differentiated thyroid cancer (Pacini and Pinchera, 1999). As thyroid-specific protein, its levels in lymph nodes are normally very low and an increased Tg level in the needle washout has been associated with metastasis of lymph nodes in patients affected by differentiated thyroid carcinoma (Giovanella et al., 2013). Its determination is currently based

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on immunometric-chemiluminescent or radioimmunometric assays (Spencer and Lopresti, 2008). The first methods require the use of enzyme-labeled monoclonal and polyclonal anti-Tg antibodies, assessing the presence of Tg by antibody-conjugated enzyme activity. Radioimmunometric assays are also based on the use of anti-Tg antibodies bearing specific radiolabels. Such techniques have lengthy incubation and washing steps, resulting excessively time-consuming. Moreover the need of labeled antibodies is a significant weakness, not allowing the real time detection of the target biomolecule and making conventional immunoassays techniques for Tg detection not easily amenable to clinical use. New effective, accurate, sensitive and rapid biosensing techniques are urgently needed.

In recent years efforts to define and optimize diagnostic and biosensing tools that incorporate such features are significantly increased. Molecular biosensors are preferred as clinical diagnostic tools than other traditional methods because of real-time measurement, rapid diagnosis, multi-target analyses, automation, and reduced costs. A few works have so far been proposed regarding Tg detection using a biosensor platform. In 2008 Choi et al. detected Tg in a cocktailed mixture of proteins by using the competitive protein adsorption/exchange reactions, namely Vroman effect. Implemented on a microfluidic system, the target protein displaced a pre-adsorbed weak-affinity protein on one surface of the device, while another pre-adsorbed high-affinity protein on an adjacent surface was not displaced. Differential measurement using surface plasmon resonance (SPR) phenomenon allowed Tg detection (Choi and Chae, 2009).

Recently Dantham et al. (2013) reported the detection of single human Tg protein molecule from the resonance frequency shift of a whispering gallery mode–nanoshell hybrid resonator upon adsorption on the nanoshell. However, although the high sensitivity of the proposed devices, the absence of a bioreceptor featuring high specificity and affinity, which can then discriminate between target and non-target molecules, prevented the use of such systems in clinical and diagnostic applications.

Moreover in the last years, the continuous demand for lower limits of detection combined with cost effectiveness and reliability features has been the driving force for the successful demonstration of optical label free biosensors with impressive figures of merit (Fan et al., 2008; Hoa et al., 2007). Relative principles of operation include SPR (Chung et al., 2006; Teramura and Iwata, 2007), interferometry (Weisser et al., 1999; Schneider et al., 1997), optical waveguide-based biosensors (Website, http://www.neosensors.com), optical ring resonators (Chao et al., 2006; Ren et al., 2007; Hanumegowda et al., 2005), fiber-based biosensors (Lee and Fauchet, 2007; Skivesen et al., 2007, Chryssis et al., 2005; DeLisa et al., 2000; Zhang et al., 2005). Among the others, fiber optic optrodes constitute a valuable platform for label biosensing because of its intrinsic biocompatibility, compact size, multiplexing capability, remote operation and easy integration in medical needles. In particular, in this work we selected optical fiber LPGs as evanescent wave-based biosensors for the measurements of local refractive changes due to molecular binding occurring at the sensor surface (Pilla et al., 2011; 2012a, 2009; Del Villar et al., 2005; Cusano et al., 2006).

An LPG consists of a periodic modulation of the RI at the core of an optical fiber that results in the coupling of the light between core and cladding modes (James and Tatam, 2003). Thanks to the huge sensitivity to surrounding refractive index (SRI) changes, LPGs represent a very promising technological platform, which can be employed in a wide number of chemical and biological applications (Chiavaioli et al., 2014; Eftimov, 2010; Baldini et al., 2012; Tripathi et al., 2012; Smietana et al., 2015; Falciai et al., 2001; Falate et al., 2005; Chen et al., 2007; Ramachandran et al., 2002). The main disadvantage of LPG biosensors experienced so far is the intrinsic transmission mode operation, which makes the optical device sensitive to bending, difficult to use in *in vitro* assays and, more importantly, creates a significant barrier for their integration in hypodermic needles. Hence, a reflection-type configuration would be desirable to combine the high sensitivity featured by this kind of devices with the unrivaled advantages of single ended optrode configurations (Garg et al., 2013; Huang et al., 2013; Consales et al., 2014; Alwis et al., 2013; Cao et al., 2013).

On this line, we present in this work the development of a reflection-type LPG biosensor able to perform the detection of thyroid cancer biomarkers in the needle washouts of fine-needle aspiration biopsies. After fabrication, the reflection-type LPG is functionalized with a hydrophobic coating of a specific bioreceptor, in our case an anti-Tg monoclonal antibody and the protein is detected in label-free experiments. Results clearly demonstrate the effectiveness and sensibility of the biosensing platform, allowing the *in vitro* detection of sub ng/ml concentrations of human purified Tg. To validate the potential translation of such LPG-based biosensor into the clinical practice, detection experiments on clinical samples have been carried out.

2. Materials and methods

2.1. Chemicals

Ethanol (EtOH), isopropyl alcohol (IPA, analytical grade 99.7), double distilled water (ddH_2O), chloroform ($CHCl_3$, analytical grade 99.9), atactic polystyrene (aPS, MW=280.000 g/mol), aqueous ammonia (NH_3 30% w/w), dextrose, human Thyroglobulin (T6830-5MG), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), 1% bovine serum albumin (BSA) solution and HRP conjugated-streptavidin (GERPN1231-100UL), chromogenic substrate, 3,3′,5,5′-Tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Milan, Italy). Anti-Tg mouse monoclonal antibody (Ab, MA5-12048) was purchased from Thermo Scientific (Monza, Italy). Potassium hydroxide solution (KOH) was purchased by J.T. Baker.

2.2. Reflection type LPGs (RT-LPG) design, fabrication and characterization

The LPGs used in this experiment were UV-written in boron doped photosensitive single-mode optical fibers (PS1250/1500, Fibercore Ltd.) using a point-to-point technique. LPGs were coated with a nano-scale high refractive index (HRI) layer of aPS in order to tune the working point of the device in the modal transition region, allowing to attain giant SRI sensitivities (of the order of thousands of nm/RIU) (Del Villar et al., 2005; Cusano et al., 2006), and at the same time provide a suitable surface for the hydrophobic adsorption of the bioreceptor.

2.2.1. LPG inscription

For the LPG inscription, the fiber was mounted on an automatic rotation stage able to continuously rotate the optical fiber during the writing grating procedure with a KrF pulsed excimer laser (LightBenth 1000, Optec, Belgium) operating at a wavelength of 248 nm. Moreover, both the rotation stage and the laser action were controlled and synchronized by a personal computer in order to select the grating pitch (translation stage step and slit dimension), the grating length (number of irradiated points) and the induced RI change (number of laser pulses per point and fluence).

2.2.2. Design of transition mode HRI-coated LPG

The matrix method with the Linearly Polarized (LP) mode approximation (Del Villar et al., 2005; Pilla et al., 2012b) was used to