

Supporting Information

In Vivo Recognition of Human Vascular Endothelial Growth Factor by Molecularly Imprinted Polymers

Alessandra Cecchini[@], Vittoria Raffa^{@#}, Francesco Canfarotta[§], Giovanni Signore^{‰+}, Sergey Piletsky[^], Michael P. MacDonald[&] and Alfred Cuschieri^{*@}*

[@]IMSaT, University of Dundee, 1 Wurzburg Loan, Dundee DD2 1FD, UK

[#]Department of Cellular and Developmental Biology, Università di Pisa, S.S. 12 Abetone e Brennero 4, 56127 Pisa, Italy

[§]MIP Diagnostics Ltd, University Road, Leicester LE1 7RH, UK

[‰]Center for Nanotechnology Innovation @NEST, Istituto Italiano di Tecnologia, Piazza San Silvestro 12, 56127 Pisa, Italy

⁺NEST, Scuola Normale Superiore, and Istituto Nanoscienze-CNR, Piazza San Silvestro 12, 56127 Pisa, Italy

[^]Department of Chemistry, University of Leicester, University Road, Leicester LE1 7RH, UK

[&]School of Science and Engineering, University of Dundee, Nethergate, DD1 4HN, UK

IMSaT, University of Dundee, 1 Wurzburg Loan, Dundee DD2 1FD, UK

vittoria.raffa@unipi.it and a.cuschieri@dundee.ac.uk

Functionalization of the solid-phase with hVEGF epitope

Glass beads (Spheriglass® 2429 CP00, 53-106 µm diameter, from Blagden Chemicals) were used as solid support for the solid-phase synthesis of nanoMIPs. The beads were first boiled in a solution of NaOH 1 M for 15 mins to activate their surface. After several washes with deionized water, the beads were rinsed with acetone and dried. Afterwards, the beads were incubated with 2% v/v 3-aminopropyltrimethoxysilane (APTES) (Sigma Aldrich) in anhydrous toluene overnight at room temperature. This step allows obtaining amino groups onto the surface of the beads. Then, unreacted APTES was washed away by rinsing the beads with acetone and methanol, and then dried. Subsequently, the amine-functionalized beads were incubated in the dark with 0.2 mg/ml succinimidyl iodoacetate (SIA) (Thermo Fisher) in dry acetonitrile for 2 hours at room temperature. After several washes with deionized water, the cysteine modified hVEGF epitope (0.2 mg/ml) was immobilized onto the surface of the SIA-beads in phosphate buffered saline (PBS, 10 mM) pH 8.3 enriched with EDTA 5 mM, overnight at room temperature in the dark. During the reaction, the haloacetyl group of SIA reacts with the thiol group of the terminal cysteine of the epitope, resulting in the coupling of the epitope onto the surface of the beads. Additionally, vancomycin-functionalized beads were prepared to produce control nanoMIPs. Vancomycin was chosen as control molecule because its isoelectric point (8.14) is similar to the one of VEGF (8.5) and the selected epitope (8.14). The synthesis of control nanoMIPs was necessary since it is not possible to obtain non-imprinted polymers with the solid-phase synthesis strategy. In this case, amine-functionalized beads were incubated in 7% v/v glutaraldehyde solution in PBS pH 7.4 for 5 hours at room temperature. The beads were then washed with deionized water and incubated in a solution of vancomycin (5 mg/ml) in PBS

pH 7.4 overnight at room temperature. Afterwards, the beads were washed with deionized water and placed in the polymerization solution for the synthesis of either nanoMIPs against hVEGF or control nanoMIPs against non-target vancomycin.

Polymerization of nanoMIPs and coupling with quantum dots

To synthesize nanoMIPs imprinted against both the epitope and control MIPs, a polymerization in PBS was performed, adapting the protocol described by Hoshino and co-workers¹. In particular, *N*-isopropylacrylamide (Sigma Aldrich) (39 mg), *N*-*tert*-butylacrylamide (Sigma Aldrich) (33 mg), *N*-(3-aminopropyl)methacrylamide hydrochloride (Polysciences, Inc.) (5.8 mg), acrylic acid (Sigma Aldrich) (2.2 μ l), and *N,N'*-methylenebisacrylamide (Sigma Aldrich) (2 mg) (Figure 2a) were dissolved in 100 ml PBS. The reaction was initiated with ammonium persulfate (APS) (Sigma Aldrich) (60 mg/ml) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Sigma Aldrich) (22 μ l) as catalyst. The whole process was carried out using a 200 ml glass bottle. Prior to initiate the polymerization process, the mixture was purged with N₂ and sonicated for 20 min. At the same time, dried functionalized beads were purged with N₂ for 20 min. The beads were then added to the polymerization solution and purged with N₂ for 2 min. The mixture of beads and polymerization solution was swirled gently and then the polymerization started by adding APS and TEMED. The polymerization was performed overnight at room temperature, after flushing the headspace of the bottle used for the reaction with N₂ for 30 seconds. The following day, the beads were washed with PBS pH 7.4 following the steps described by Canfarotta and co-workers² by using a 60 ml solid-phase extraction (SPE) cartridge with a frit of 20 μ m porosity. This is a critical step to remove all the unreacted species as well as low-affinity imprinted polymers. Afterwards, the high-affinity nanoMIPs were eluted at 65°C with deionized water. The solutions of high-affinity nanoMIPs for both the epitope and

vancomycin were washed with fresh deionized water and concentrated with 30 kDa molecular weight cutoff (Amicon Ultra, Merck Millipore) centrifugal dialysis cartridge. Finally, the final aliquot of hVEGF nanoMIPs and the control nanoMIPs were coupled to commercially available QDs (PlasmaChem), specifically with carboxyl functionalized CdTe core 710 nm emitting quantum dots. For the coupling, low binding Eppendorf vials were exploited to maximize the interaction between the QDs and the nanoMIPs. A volume of 110 μl of a 10 μM solution of quantum dots was added to a solution of 400 μl of DMF, 1 μl of 100 mM *N*-hydroxysulfosuccinimide and 0.5 μl *N,N'*-diisopropylcarbodiimide for the activation of the carboxylic groups of the QDs. The quantum dots were incubated for 20 min at room temperature. Then, 500 μl of 30 pM solution of nanoMIPs (either hVEGF nanoMIPs or control nanoMIPs) in water and 10 μl of PBS were added to the quantum dot solution, and incubated on the mixer overnight at 6°C. Afterwards, the unreacted chemical species and QDs were removed via six sequential washes with deionized water and centrifugations in low binding Eppendorf vials. The final nanoprobe, called QD-MIPs in the case of hVEGF and QD-nips for vancomycin, were suspended initially in 1 ml of deionized water for the measurement of size, concentration and fluorescence, and finally in 40 μl of deionized water for the injection in zebrafish embryos.

Size measurements: DLS and NTA

The size of QD-MIPs and QD-nips was measured with a Zetasizer Nano (Nano-S) particle-size analyzer from Malvern Instruments Ltd (UK). One milliliter of QD-MIPs in distilled water was sonicated for 2 min and then the size was analyzed at 25 °C in a 3 cm^3 disposable polystyrene cuvette. The instrument automatically sets the parameters for the measurements, and the final hydrodynamic diameter was obtained from the mean of 5 sequential measurements of the same sample. Compared to DLS, which gives estimated hydrodynamic diameter of the nanoparticles,

NTA allows obtaining more precise information on the size. In fact, DLS measures the light scattered from each nanoparticle in the sample giving an average measurement. It should be noted that a smaller particle scatters less light than a larger particle (the intensity of the scattering is proportional to the sixth power of the particle diameter); therefore, the scattered light of the latter can cover the signal from smaller particles. Hence, the particle size distribution measured by DLS could be shifted towards bigger sizes compared with that measured by NTA. NTA measurements were performed with Nanosight NS300 from Malvern Instruments Ltd (UK) to measure the size of non-functionalized MIPs imprinted against hVEGF epitope and hybrid nanoparticles QD-MIPs (embedded) and QD-MIPs (attached). One milliliter of each sample in distilled water was sonicated for 2 min and then injected in the instrument chamber for the measurement. Particularly, 5 sequential measurements of 60 seconds were performed at 25 °C.

Z potential and concentration measurements

Z potential of free QDs, MIPs and hybrid nanoparticles QD-MIPs and QD-nips was measured with a Zetasizer NanoZS90, available at Scuola Normale Superiore - Laboratorio NEST, Italy, as well as the concentration of QD-MIPs and QD-nips was evaluated. The concentration was calculated by measuring the number of particles per liter of the solution of nanoprobess, and by multiplying this number by the Avogadro's number.

Transmission electron microscopy

For transmission electron microscopy (TEM) analysis, QDs, QD-MIPs (embedded) and QD-MIPs (attached) samples were suspended in distilled water, placed on copper grids and dried overnight. All samples were examined under JEOL 100X1 TEM.

Fluorescence measurements

The Fluorescence intensity of QD-MIPs was measured with a Fluoromax-2 fluorimeter from

Horiba Scientific, UK. The measurements were performed at room temperature in 1 ml quartz cuvettes of 10 mm path length. The excitation wavelength was set at 405 nm and the emission signal was collected from 540 nm to 800 nm. All the measurements were obtained for samples suspended in water. Since it was observed that the coupling affects the emission wavelength of the of the QDs exploited for this study, a solution of free QDs was used as reference.

Affinity and specificity assays

To assess the affinity of the QD-MIPs for the template, SIA Au SPR gold chips (GE Healthcare) were exploited. Gold chips were first cleaned by using Emitech K1050X Plasma Cleaner (Emitech) at 50 W for 5 min. Afterwards, chips were functionalized with a solution of 0.2 mg/ml cysteamine (Sigma Aldrich) in ethanol, overnight at room temperature in sealed vials. The functionalization of the chips with cysteamine allows obtaining amine groups onto the surface of each chip, for the coupling with the carboxylic groups of free QDs and QD-MIPs. Chips were then rinsed with deionized water and ethanol and dried under N₂. The chips were assembled on the holder following the manufacturer instructions and then docked onto the SPR instrument (Biacore 3000, GE Healthcare). For this set of experiments, QD-MIPs and free QDs were immobilized online onto different flow cells of the chip, by injecting a solution of QD-MIPs or QDs in water with 5 mg/ml EDC. Three injections of 100 µl of the two different ligands were performed onto the chip at 20 µl/min until 1000 response units were reached. Then, 100 µl of carbonate-bicarbonate buffer (Sigma Aldrich) were injected onto the chip. Six different concentrations of the analyte, either the epitope or vancomycin, ranging from 97 pM to 100 nM, were injected. The same procedure was used for EGFR epitope, that was used as protein control. EGFR is endogenously produced in zebrafish and it is known to be involved in angiogenesis, therefore it could be one of the proteins exposed to QD-MIPs in xenotransplanted zebrafish

embryos. Additionally, concentrations of vancomycin were increased up to 1 μM (ranging from 10 nM to 1 μM). All the experiments were performed in deionized water. Each concentration was injected for 7 min at flow rate 25 $\mu\text{l}/\text{min}$ and the dissociation time was set at 2 min. The dissociation constants were obtained with the BiaEvaluation software v4.1. Data were analyzed by fitting the data of QD-MIPs after subtracting the signal of QDs and assuming a 1:1 Langmuir binding model. For the binding assessment, the same procedure for the immobilization of QD-MIPs and QDs was followed as explained above. A solution of 40 nM recombinant hVEGF (produced by Prof Ballmer's group) reconstituted in deionized water was injected onto the chip for 7 min at flow rate 25 $\mu\text{l}/\text{min}$ and the dissociation time was set at 2 min. All experiments were performed twice.

Preparation of the *in vivo* tumor model in zebrafish and injection of nanoprobe

For the creation of zebrafish embryo tumor models, two cell lines were chosen: (i) human malignant melanoma WM-266 cells, known to overexpress hVEGF - hVEGF (+) model - and (ii) the human melanoma A-375 cells, stably transfected with GFP and resistance to puromycin, as control cells - hVEGF (-) model. Both cell lines were cultured in complemented high glucose DMEM under standard conditions (5% CO_2 , 37°C). A-375 cells were cultured in DMEM further complemented with puromycin 2 $\mu\text{g}/\text{ml}$ for the selection of GFP positive A-375. Instead, WM-266 cells were labeled with CM-DiI dye before injection. When WM-266 cells were approximately 80% confluent, they were trypsinized, centrifuged (3000 g for 5 min at room temperature), re-suspended in PBS and incubated with CM-DiI, used in accordance to the manufacturer protocol, for 15 min at 37°C and finally 15 min at 4°C. Before injection, WM-266 cells were centrifuged in PBS 3 times. Finally, both WM-266 and A-375 cells (previously trypsinized once were approximately 80% confluent) were re-suspended in PBS supplemented

with fetal bovine serum (FBS) 10% v/v. Either cell lines were injected with heat-pulled needle and manual injector (Figure S5a) in one side of the yolk of 48 hpf zebrafish embryos (Figure S5b – c). Twenty-four hours after the injection of cells, the embryos were screened with epifluorescence microscope and injected with the two solutions of nanoprobe in the other side of the yolk. Particularly, we obtained three groups: (i) hVEGF (+) model injected with hVEGF QD-MIPs; (ii) hVEGF (+) model injected with vancomycin QD-nips; (iii) hVEGF (-) model injected with QD-MIPs. All volumes injected were 30 nl starting from 30 pM solutions of nanoprobe. For each group, at least 30 embryos were injected.

Inductively coupled plasma mass spectrometry (ICP-MS) quantification

ICP-MS measurements were performed in accordance with established protocols. Ten 48 hpf zebrafish embryos were injected with QD-MIPs, sacrificed at different incubation times: 0 hours, 2.5 hours and overnight (20 hours). The embryos were dissolved in 200 μ L of nitric acid (Trace analysis grade), and the solution was then digested in a microwave reactor (Discover SP-D, CEM) for 20 min at 200 °C. After digestion, the sample was diluted to 2 ml with water for trace analysis and analyzed by ICP-MS (Agilent Technologies 7700 Series ICP-MS). Cadmium content in digested samples was quantified by ICP-MS. The Cadmium content in each embryo was determined by subtracting the blank and normalizing the signal for the number of injected embryos and the signal at incubation time 0 min.

Preparation of samples for confocal microscopy

Seven hours after injection of QD-MIPs, the embryos were fixed by immersion in a 4% paraformaldehyde (PFA) solution for 30 min at room temperature. Following fixation process, PFA was decanted and embryos gently and quickly washed with PBS. To ensure protection against frost damage during storage at -80°C, the embryos were immersed in a 20% sucrose

solution for 2 hours at room temperature. Thereafter, the embryos were placed in molds for inclusion in optimal cutting temperature compound (OCT), the sucrose solution removed, OCT added to cover the embryos in the mold, the embryos were aligned and the molds placed first in EtOH at -80°C for 10 min and then finally stored at -80°C for at least 1 hour. For the sectioning, a cryostat-microtome was used to obtain 40 µm thick sagittal sections. Each slice was collected by using a positively-charged microscope slide. Once dried at room temperature, all sections were then covered with a few drops of Polymount solution and cover slips, placed under fume hood for 1 hour and finally stored overnight at 4°C.

Confocal microscopy

The sections were imaged with confocal microscope Leica SP2. Notably, this microscope can detect wavelengths up to 690 nm. Although the instrument does not allow the collection of the whole emission spectrum of the QDs, it can detect the increasing emission peak. The nanoprobe were excited with 405 nm laser whereas A-375 expressing GFP with 488 nm laser and WM-266 labeled with DiI with 561 nm laser. In the case of the nanoprobe, emission spectrum data were acquired by exciting with 405 nm laser and acquiring the spectrum from 600 nm to 750 nm, with wavelength steps of 5 nm.

Evaluation of the images and statistical analysis

The statistical analysis software GraphPad Prism 5 was exploited for a better understanding of the localization patterns of the nanoprobe and the evaluation of overall nanoprobe-cell distances. For the analysis of these data, only embryos in which the number of 'red spots' was equal or higher than ten were included in the analysis. The distances were calculated in a standardized manner by using a fixed intensity threshold for the nanoprobe and Fiji 'analyze nanoparticles' and 'measure' plugins. The coordinates (in µm) of the centers of mass of both the

nanoprobes and cells, obtained with ‘measure’ plugin, where then copied in Office Excel. Particularly, considering one nanoprobe, centers of mass of each of the closest cells were obtained. For each nanoprobe, we considered only the shortest distance from the closest cell as parameter for final analysis. Finally, the nanoprobe-cell distances were calculated with Pythagoras’s equation. The procedure is schematized in Figure S6. Moreover, ANOVA Bonferroni analysis was exploited to evaluate the data of the means of nanoprobe-cell distances ($n \geq 7$).

REFERENCES

- (1) Hoshino, Y.; Kodama, T.; Okahata, Y.; Shea, K. J. *JACS*, **2008**, *130*, 15242–15243.
- (2) Canfarotta, F.; Poma, A.; Guerreiro, A.; Piletsky, S. A. *Nat. Protoc.* **2016**, *11*, 443–455.