Electronic Supplementary Material (ESI) for Analytical Methods.

Appendix

A.1. Reagents

Recombinant human leptin protein as standard, anti-leptin antibody produced in rabbit (Abcam, Cambridge, United Kingdom), progesterone, cortisol, estradiole, human albumin, bovine serum albumin (BSA), cysteamine hydrochloride, N-ethyl-N'-(3-dimethylaminopropyl) carbodimide (EDC), N-hydroxysuccimide (NHS) (Sigma-Aldrich, Steinheim, Germany), photopolimer ELPEMER SD 2054 and hydrophobic protective paint SD 2368UV SG-DG (Peters, Kempen, Germany) were used, as well as absolute ethanol, acetic acid, hydrochloric acid, sodium hydroxide, sodium chloride, sodium carbonate, sodium acetate (POCh, Gliwice, Poland). HBS-ES buffer pH=7.4 (0.01 M HEPES, 0.15 M sodium chloride, 0.005% Tween 20, 3 mM EDTA), Phosphate Buffered Saline (PBS) pH=7.4, carbonate buffer pH=8.5 (BIOMED, Lublin, Poland,) were used as received. Aqueous solutions were prepared with milliQ water (Simplicity® Millipore). Argon N 5.0 with a content Ar ≥ 99,999% was used (AIR LIQUIDE Polska Sp.z o.o., Poland). Standard ELISA kit for leptin determination (Abcam, Cambridge, United Kingdom) was used.

A.2. Biological samples

Blood samples were taken from children with malnutrition on admission to the L. Zamenhof Children's Clinical Hospital of the Medical University of Bialystok, Pediatric Surgery Department (Bialystok, Poland). The control group consisted of healthy, normally nourished children admitted for planned herniotomy. The blood samples were collected from the median cubital vein using EDTA as an anticoagulant.

Two ml of blood was centrifuged (1000 x g) for 15 min and filtered three times to separate the plasma from the blood cells. The plasma samples were frozen and stored at –70 °C until further use. The samples were diluted twice with PBS buffer directly prior to measurement.

A.3. Chip preparation and antibody immobilization

To bind leptin from the sample it was necessary to immobilize a suitable biological receptor layer, which consisted of leptin-specific rabbit antibody. The immobilization consisted of several steps. The first was the formation of a cysteamine monolayer. For this purpose, the gold chip was rinsed with absolute ethanol and water, dried in a stream of argon, and immersed in

20 mM cysteamine ethanolic solutions for a minimum of 18 h. After this time, the chip was rinsed again with ethanol and water and dried with a stream of argon. Cysteamine is now immobilized on the sensor surface with the amine group on top. The prepared chip with cysteamine was stable for one month.

The second step is the binding of the antibody to the cysteamine by covalent immobilization. To form amide bonds between carboxy groups of the antibody and amine groups of the linker, 25 µL of antibody solution was activated with 125 µL of 50 mM NHS and 125 µL of 200 mM EDC in carbonate buffer (pH=8.5), and was then placed on the amine-modified surface. The optimum antibody concentration was determined experimentally to be 60 ng mL^{-1}. 2.5 µL of antibody solution was placed on the amine-modified surface and incubated at 37 °C for 1 hour. The biosensor prepared in this way was rinsed five times with PBS buffer. 1 mg mL^{-1} BSA in PBS was placed on the chip to minimize nonspecific adsorption on the surface, and it was rinsed with PBS buffer. To deactivate any remaining amine groups, a mixture of sodium acetate (50 µL, 5 mM), NHS (250 µL, 50 mM), EDC (250 µL, 200 mM) and carbonate buffer (100 µL, pH=8.5) was used. The chip was then rinsed with PBS buffer and water and dried with a stream of argon. The prepared immunosensor was stable for 24 hours.

During preparation of the biosensor, the surface was observed by Atomic Force Microscopy (AFM) (Ntegra Prima scanning probe microscope, NT-MDT, Russia). AFM measurements were performed after the creation of successive layers. This was done to confirm that the described stages of the creation of successive layers on the biosensor surface had in fact taken place. Subsequent images are shown in Fig. A.1.

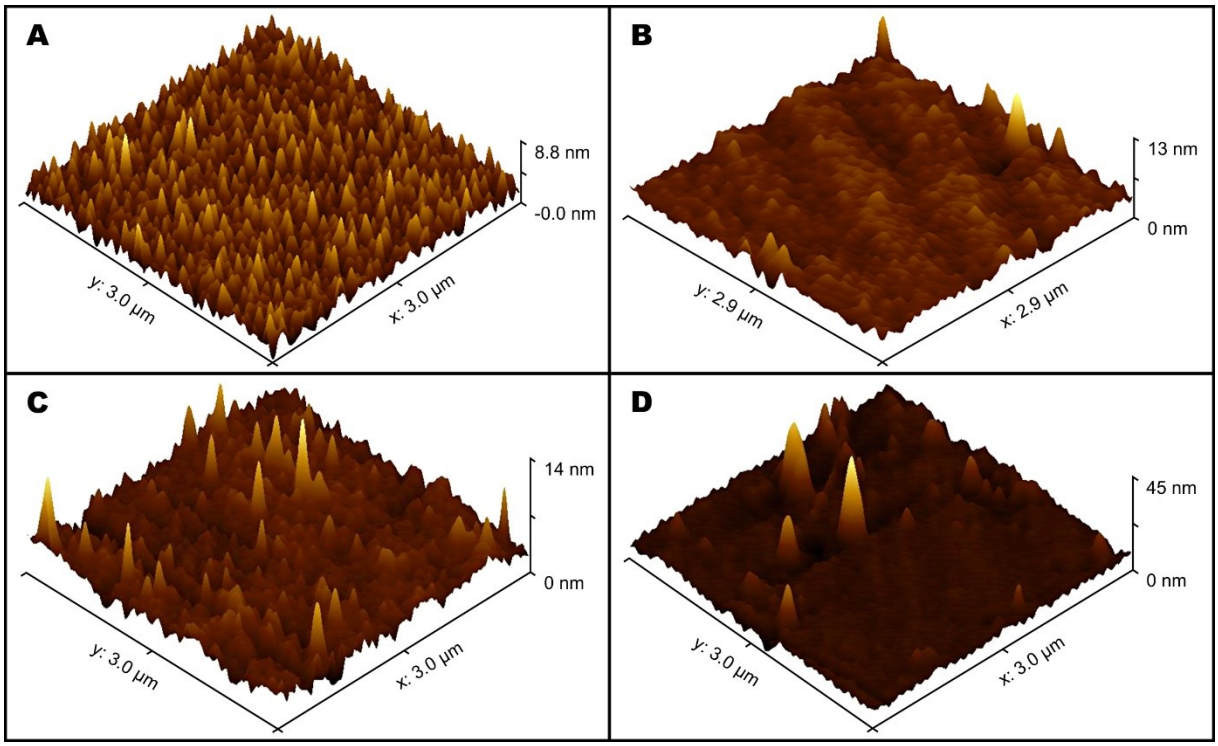

Fig. A1. AMF images of bare gold (A), after cysteamine immobilization (B), as (B) after antibody immobilization (C), and as (C) after interaction with leptin (D).

A.4. Optimization of measurement conditions. Selection of optimal concentration of rabbit anti-leptin antibody

The dependence of the analytical signal of leptin on antibody concentration was tested at a constant leptin concentration equal to 5 ng mL^{-1} within an antibody concentration range between 2 and 100 ng mL^{-1}. The experiment was performed as described above (A.3). The results are shown in Fig. A.2.

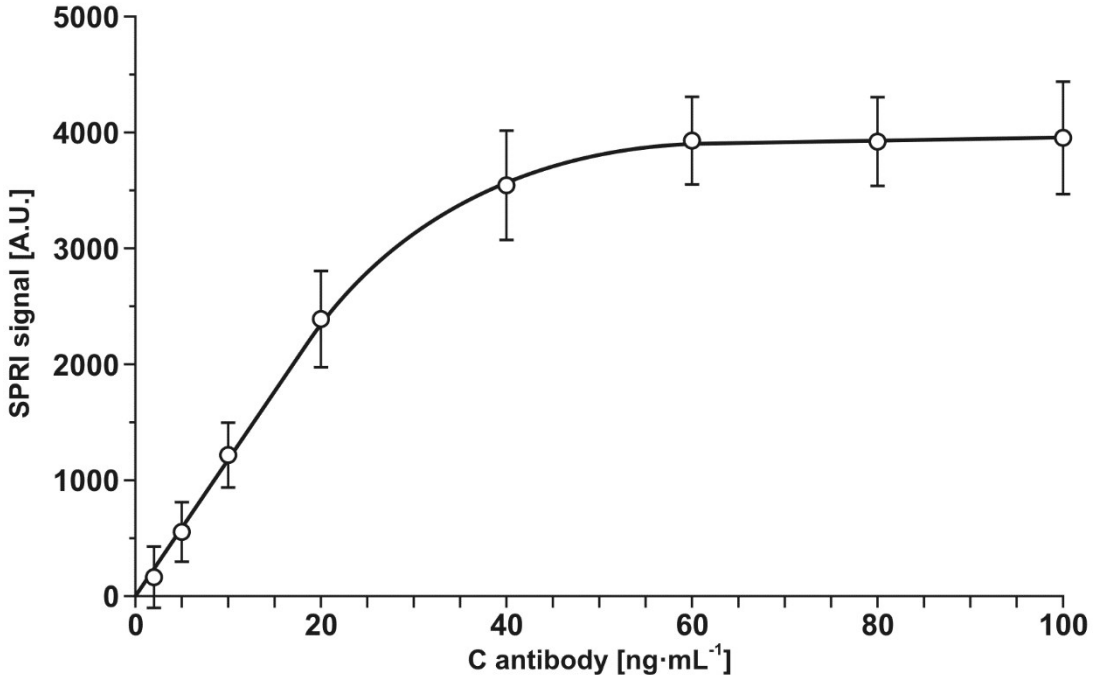

Fig. A 2. Dependence of SPRI signal (Arbitrary Units) on anti-human leptin antibody concentration. Leptin concentration: 5 ng mL^{-1}; pH = 7.4. Error bars were calculated for 12 independent measurements for each concentration at a 95% confidence level.

The formation of a plateau on the graph is due to saturation of the chip surface with the antibody. An antibody co ncentration of 60 ng mL^{-1} was selected for further experiments.