

An ultra-sensitive SPR immunosensor for quantitative determination of human cartilage oligomeric matrix protein biomarker

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Keywords: SPR immunosensor, COMP, Quantum dots, Dual sandwich-type signal amplification, Biotin–streptavidin interaction

ABSTRACT

This paper reports the development of a novel surface plasmon resonance (SPR) immunosensor for ultra-sensitive quantitative determination of human articular cartilage oligomeric matrix protein (COMP), a major component of the extracellular matrix and an exploratory biomarker. Capture antibodies against human COMP (anti-COMP_{16F12}) were covalently immobilized on an 11-mercaptopundecanoic acid (11-MUA) self-assembled monolayer (SAM)-coated SPR sensor disk and a dual sandwich-type signal amplification strategy using biotinylated detection antibodies against COMP (anti-COMP_{17C10-biot}) and streptavidin-conjugated quantum dots (SAv-QDs) were used for the development of an immunosensor. The binding of high-mass SAv-QDs via biotin-streptavidin interaction to the surface of the immunosensor resulted in a drastic increase in the sensitivity. The developed immunosensor was able to detect concentrations of COMP in a range from 2.80 to 680.54 fM with a limit of detection (LOD) and a limit of quantification (LOQ) of 0.15 and 0.50 fM, respectively. The immunosensor exhibited good repeatability (relative standard deviation (RSD) 8.05%) and reproducibility (RSD 9.88%) as well as excellent operational stability (2.14 % decrease in SPR signal after 13 days). In addition, the analysis of secretomes of human knee articular cartilage explants from patients with osteoarthritis revealed that the immunosensor has good accuracy (analytical error less than 5 %). These results indicate that the immunosensor developed may be suitable for quantitative determination of COMP derived from articular cartilage and other synovial joint tissues in clinical studies.

1. INTRODUCTION

The operation of SPR biosensors, pioneered by Liedberg et al. (1983) and Flanagan and Pantell (1984), is based on the detection of changes in the refractive index of the environment very close (approximately 300 nm) to the sensor surface. Biosensors that exploit the specific immunoreaction of antigen and antibody, so-called immunosensors, are currently one of the most commonly developed and studied SPR biosensors. Currently, SPR immunosensors are promising analytical tool for environmental, food and beverage analysis, industrial and homeland security applications (Ravi Shankaran and Miura, 2007), as well as clinical (Mariani and Minunni, 2014) and medical diagnostics (Masson, 2017). They are suitable for the sensitive and accurate quantification of a wide range of analytes in small volume samples containing high levels of extraneous interfering substances. Furthermore, the analysis often takes only a few minutes, and multiple analyses can be performed after proper regeneration of the sensor surface. Compared to traditional immunoassay methods such as enzyme-linked immunosorbent assay or radioimmunoassay, the main advantage of SPR immunosensors is that they allow real-time and label-free detection (Kausaite-Minkstimiene et al., 2013). However, while the label-free direct detection format is very attractive as it allows for rapid and cost-effective analysis, it has the disadvantage of low sensitivity when the molecular weight of the analyte is low. This is because the change in refractive index caused by the interaction between a ligand immobilized on a surface of the SPR sensor and an analyte present in a solution depends on molecular weight of analyte. The lower the molecular weight, the smaller the change in refractive index. Therefore, the direct detection format is generally not suitable for the quantification of low molecular weight analytes as well as relatively high molecular weight analytes at picomolar or lower concentrations. In such cases, indirect sandwich or competitive inhibition assays are usually used to quantitate analytes (Homola and Piliarik, 2006). In addition, detection antibodies and competing molecules are often labelled with high-mass labels to further increase sensitivity.

In recent years, various plasmonic nanoparticles, especially those made of silver or gold, and non-plasmonic nanomaterials such as carbon nanotubes, magnetic or silica nanoparticles have been used as high-mass labels. The use of these nanomaterials results in a much larger change in refractive index and thus significantly improves the sensitivity of the SPR immunosensor. In the case of plasmonic nanoparticles, the increase in sensitivity of the immunosensor is additionally determined by the coupling between surface plasmon polaritons from the SPR sensor and localized surface plasmons from the nanoparticles (Zeng et al., 2017). For example, the use of multi-walled carbon nanotubes conjugated with detection antibodies against Tau protein allowed to increase the response of the SPR immunosensor up to 100 times compared to direct detection and conventional sandwich-type

detection, and to reduce a LOD to 125 pM (Lisi et al., 2017). A similar way of signal amplification using silica nanoparticles was demonstrated for the quantitative determination of staphylococcal enterotoxin G. Antibody-coated silica nanoparticles induced a higher immunosensor response than free detection antibodies (Belen et al., 2021). Jia et al. (2018) demonstrated that conjugates of antibodies against 17 β -estradiol and magnetic nanoparticles enhance the analytical signal of an SPR immunosensor operating in a competitive inhibition immunoassay format. The use of magnetic nanoparticles allowed to reduce the LOD for 17 β -estradiol quantification from 3.24 ng/mL to 0.81 ng/ml. Kausaite-Minkstimiene et al. (2022) demonstrated the use of signal amplification strategy based on antibody-functionalized gold-coated magnetic nanoparticles for ultra-sensitive and quantitative detection of CD5 biomarker through a sandwich-type immunoassay format. Compared to the direct detection format, LOD was reduced from 1.04 nM to 8.31 fM. Similar to gold-coated magnetic nanoparticles, conventional gold nanoparticles have also been used to enhance the sensitivity of SPR immunosensors (Ermini et al., 2019; Pawula et al., 2016). Eletxigerra et al. (2016) used SAv decorated gold nanoparticles and a dual sandwich-type amplification strategy to develop an SPR immunosensor for the quantitative determination of Epidermal Growth Receptor Factor 2. Because the immunosensor response was greatly enhanced, the LOD in 50% human serum was 180 pg/mL, which is 83-fold below the clinical threshold. Since silver is more sensitive to harsh environmental conditions and is characterized by less rich surface chemistry, gold nanoparticles remain the first choice, but silver nanoparticles can also be suitable for increasing the sensitivity of SPR immunosensors (Paul et al., 2011).

QDs are semiconductor nanocrystals composed of approximately 100 to 10,000 atoms, exhibiting size- and chemical composition-dependent fluorescence properties (Maxwell et al., 2020). Due to their exceptional physical and chemical properties, they have attracted great interest from scientists and have been widely used in medical diagnostics, immunology, and biomedical research for sensing and imaging purposes (Zhou et al., 2015). More recently, several SPR immunosensors have been developed with greatly enhanced sensitivity using QDs and biomolecule conjugates as high-mass signal-amplifying labels (Anderson et al., 2013; Wang et al., 2016). Inspired by these research advances, we used QDs to develop an SPR immunosensor for the quantitative determination of COMP, also known as thrombospondin 5, a pentameric, non-collagenous glycoprotein (Verma and Dalal, 2013). It is one of the main proteins of cartilage extracellular matrix (ECM) and its turnover and catabolic degradation has led to a number of studies to explore its potential as a biomarker of osteoarthritis (OA) (Hosnijeh et al., 2015). COMP is detected in serum and synovial fluid, and increases in clinical and animal models of OA in relation to the mechanical load, severity of disease

as well as the number of joints affected in clinical and animal models (Piscoya et al., 2005). COMP is also expressed in many other connective tissues and is implicated in several other inflammatory joint disorders such as rheumatoid arthritis, reactive arthritis, psoriatic arthritis, or acute trauma (Lorenzo et al., 2017). Despite the potential for COMP being an important biomarker, to our knowledge, no SPR immunosensor has yet been developed for its quantification.

This paper describes the development and investigation of SPR immunosensor for ultra-sensitive quantitative determination of COMP biomarker. Anti-COMP_{16F12} immobilized on 11-MUA SAM-coated SPR sensor disk and a dual sandwich-type signal amplification strategy using anti-COMP_{17C10}-biot and SAV-QDs were applied to develop this immunosensor. Anti-COMP_{16F12} concentration and immunosensor surface regeneration conditions were optimized to achieve the best performance. The ability of the developed immunosensor to detect COMP was compared with that of immunosensors based on a direct detection format and a conventional sandwich-type detection format. The main analytical characteristics were also examined. Finally, the ability of the developed immunosensor to detect COMP was tested by analysing knee articular cartilage explant secretome from patients with OA.

2. EXPERIMENTAL SECTION

2.1. Materials and reagents

Human COMP (molecular weight 82.4 kDa, >90% purity as determined by densitometric image analysis), anti-COMP_{16F12} (monoclonal mouse IgG1 clone 16F12, purified on an affinity chromatography column with immobilized protein G), anti-COMP_{17C10} (monoclonal mouse IgG1 clone 17C10, purified on an affinity chromatography column with immobilized protein G) and human COMP enzyme-linked immunosorbent assay (ELISA) kit were purchased from BioVendor (Brno, Czech Republic). EZ-Link™ Sulfo-NHS-Biotin antibody biotinylation kit was obtained from Thermo Fisher Scientific (Pittsburgh, Pennsylvania, USA). 1 μM solution of 15–20 nm in size SAV-QDs (Qdot™ 625 streptavidin conjugate) was purchased from Invitrogen by Thermo Fisher Scientific (Waltham, Massachusetts, USA). 11-MUA (98% purity, CAS No.: 71310-21-9), sodium dodecylsulfate (SDS, ACS reagent, ≥99.0% purity, CAS No.: 151-21-3), sodium acetate trihydrate (NaAc, ACS reagent, ≥99 % purity, CAS No.: 6131-90-4), methanol (anhydrous, 99.8% purity, CAS No.: 67-56-1), hexane (anhydrous, 95% purity, CAS No.: 110-54-3), penicillin-streptomycin (bioreagent, suitable for cell culture), L-proline (USP testing specifications, suitable for cell culture, CAS No.: 147-85-3), dexamethasone (≥97% purity, bioreagent, suitable for cell culture, CAS No.:

50-02-2) and L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate ($\geq 95\%$ purity, CAS No.: 1713265-25-8) were obtained from Sigma-Aldrich (Steinheim, Germany). EDC hydrochloride ($\geq 99.0\%$ purity, CAS No.: 25952-53-8), acetic acid (100%, Ph. Eur., extra pure, CAS No.: 64-19-7) and phosphate buffered saline tablets (PBS, for biochemistry and molecular biology) were received from Carl Roth (Karlsruhe, Germany). Dulbecco's Modified Eagle Medium (DMEM) and Insulin-Transferrin-Selenium (ITS) were obtained from Gibco™ (Waltham, Massachusetts, USA). NHS (99% purity, CAS No.: 6066-82-6), ethanolamine ($\leq 100\%$ purity, CAS No.: 141-43-5) and hydrochloric acid (HCl, fuming, 37% purity, CAS No.: 7647-01-0) were purchased from Merck (Darmstadt, Germany). Glycine (for analysis, CAS No.: 56-40-6) were obtained from AppliChem (Karlsruhe, Germany), refractive index ($n = 1.518$) matching fluid – from Cargille Labs (Cedar Grove, New Jersey, USA), sodium hydroxide (NaOH, pellets, Pharmed®, Ph Eur, BP, NF, CAS No.: 1310-73-2) – from Scharlab S.L. (Sentmenat, Spain). Solution of 11-MUA was prepared in methanol, other solutions needed for experiments were prepared using ultra-high quality (UHQ) water, which was obtained using the DEMIWA rosa 5 water purification system (WATEK, Czech Republic).

2.2. Biotinylation of anti-COMP_{17C10} antibodies

Biotinylation of detection anti-COMP_{17C10} antibodies was performed using Sulfo-NHS-Biotin biotinylation kit. Firstly, 200 μL solution of anti-COMP_{17C10} antibodies (0.5 mg/mL) was mixed with 58.9 μL of freshly prepared 0.23 mM Sulfo-NHS-Biotin solution in 10 mM PBS (pH 7.4). After 30 min incubation at room temperature the dialysis was performed to exclude free Sulfo-NHS-Biotin molecules. The dialysis procedure was done against 10 mM PBS pH 7.4 solution using Slide-A-Lyzer™ MINI Dialysis Device (20 K MWCO, Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA) under stirring. Relative sample to PBS volume ratio was at least 1:1000. The PBS (pH 7.4) was refreshed after 2 h and dialysis was continually performed overnight at 4 °C. Lastly, solution of anti-COMP_{17C10}-biot antibodies was poured into microcentrifuge tube and stored at 4 °C between experiments. The concentration of anti-COMP_{17C10}-biot was calculated by knowing the concentration and volume of the anti-COMP_{17C10} solution taken for the biotinylation procedure and by estimating the final volume of the anti-COMP_{17C10}-biot solution. The calculated anti-COMP_{17C10}-biot concentration was 0.474 mg/mL.

2.3. SPR sensor disk preparation and anti-COMP_{16F12} immobilization

The gold surface of the SPR sensor disk (SD AU, XanTec bioanalytics GmbH, Münster, Germany) was cleaned by incubation for 30 min in methanol and 2 min in hexane. The sensor disk was then washed with UHQ water, immersed in a 1 mM 11-MUA solution in methanol, and kept in it for 24 h at room temperature. After the 11-MUA SAM was formed on the surface of the sensor disk (Au/11-MUA), the sensor disk was washed with methanol and UHQ water, and dried in laboratory environment. Au/11-MUA was then deposited through a refractive-index-matched liquid onto a glass half-cylindrical prism mounted on a slider. The slider was placed in a double channel SPR analyser Autolab Esprit (Metrohm Autolab BV, Utrecht, The Netherlands) and an SPR cuvette with a surface area of 7.9 mm² per channel was screwed on. To stabilize/rehydrate the Au/11-MUA surface, it was exposed to 10 mM NaAc buffer (pH 4.5) and solution composed of 10 mM NaOH and 0.5% SDS alternately every 2 min until a stable baseline was obtained. After the stabilization/rehydration procedure, the Au/11-MUA surface was exposed to 10 mM NaAc buffer (pH 4.5) for 200 s, followed by a 1:1 mixture of 0.4 M EDC and 0.1 M NHS for 600 s. After removing the EDC/NHS mixture from the cuvette by washing it with 10 mM NaAc buffer (pH 4.5), the Au/11-MUA was exposed to anti-COMP_{16F12} solution in 10 mM NaAc buffer (pH 4.5) for 1800 s. Anti-COMP_{16F12} were immobilized by the formation of a strong amide bond between the primary amino groups of the antibodies and the carboxyl groups of 11-MUA (Au/11-MUA–anti-COMP_{16F12}). After removing unbound anti-COMP_{16F12} from the cuvette by washing it with 10 mM NaAc buffer (pH 4.5), Au/11-MUA–anti-COMP_{16F12} was exposed to 1 M ethanolamine solution (pH 8.5) for 600 s. Finally, Au/11-MUA–anti-COMP_{16F12} was exposed to 10 mM PBS (pH 7.4) and solution composed of 10 mM NaOH and 0.5% SDS alternately every 2 min until a stable baseline was obtained.

2.4. Interaction between immobilized anti-COMP_{16F12} and COMP and regeneration of Au/11-MUA–anti-COMP_{16F12}

First, Au/11-MUA–anti-COMP_{16F12} was exposed to 10 mM PBS (pH 7.4) for 200 s, and then COMP solution in 10 mM PBS (pH 7.4) was injected into one channel (measurement channel) of the SPR cuvette. Pure PBS was injected into the other channel of the SPR cuvette for a negative control (reference channel). The interaction between immobilized anti-COMP_{16F12} and COMP in solution was recorded for 600 s, followed by dissociation in 10 mM PBS (pH 7.4) for 100 s. Au/11-MUA–anti-COMP_{16F12} was then regenerated by exposure immunosensor to a solution consisting of 10 mM NaOH and 0.5% SDS for 200 s (regeneration solution). Finally, a baseline was restored by exposure of Au/11-MUA–anti-COMP_{16F12} in 10 mM PBS (pH 7.4). The difference between the measurement

and the reference channels was used to estimate the SPR signal generated during the anti-COMP_{16F12}/COMP interaction.

2.5. Sandwich-type COMP detection using anti-COMP^{17C10}

After the formation of the anti-COMP_{16F12}/COMP immune complex (Au/11-MUA–anti-COMP_{16F12}/COMP), it was exposed to 10 mM PBS (pH 7.4) for 60 s, and then 100.19 nM solution of detection anti-COMP_{17C10} antibodies in 10 mM PBS (pH 7.4) was injected into measurement channel of the SPR cuvette. Pure PBS was injected into the reference channel of the SPR cuvette. The interaction between anti-COMP_{16F12}/COMP and anti-COMP_{17C10} was carried out for 600 s, followed by dissociation in 10 mM PBS (pH 7.4) for 100 s. Au/11-MUA-anti-COMP_{16F12} was then regenerated by exposing the immunosensor surface to a regeneration solution for 200 s, followed by restoring the baseline by exposure to 10 mM PBS (pH 7.4). The difference between the measurement and the reference channels was used to estimate the SPR signal generated during the anti-COMP_{16F12}/COMP and anti-COMP_{17C10} interaction.

2.6. Dual sandwich-type COMP detection using anti-COMP_{17C10}-biot and SAV–QDs

After the formation of the anti-COMP_{16F12}/COMP immune complex, Au/11-MUA–anti-COMP_{16F12}/COMP was exposed to 10 mM PBS (pH 7.4) for 60 s, and then 55.37 nM solution of detection anti-COMP_{17C10}-biot antibodies in 10 mM PBS (pH 7.4) was injected into measurement channel of the SPR cuvette. Pure PBS was injected into the reference channel of the SPR cuvette. The interaction between anti-COMP_{16F12}/COMP and anti-COMP^{17C10-biot} (Au/11-MUA–anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot) was carried out for 600 s, followed by dissociation in 10 mM PBS (pH 7.4) for 60 s. 10.00 nM solution of SAV–QDs in 10 mM PBS (pH 7.4) was then injected into measurement channel of the SPR cuvette. Pure PBS was injected into the reference channel of the SPR cuvette. The interaction between anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot and SAV–QDs was carried out for 600 s, followed by dissociation in 10 mM PBS (pH 7.4) for 60 s. Finally, Au/11-MUA–anti-COMP_{16F12} was regenerated by exposing the immunosensor surface to a regeneration solution for 200 s, and the baseline was restored by exposure to 10 mM PBS (pH 7.4). The difference between the measurement and the reference channels was used to estimate the SPR signal generated during the anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot and SAV–QDs interaction.

2.7. Studies of SAV-QDs non-specific sorption

The Au/11-MUA-anti-COMP_{16F12} surface was firstly exposed to 10 mM PBS (pH 7.4) for 200 s to establish a stable baseline, then the SAV-QDs solution (10.0, 20.0 or 30.0 nM) was injected into the measurement channel of the SPR cuvette. Pure PBS was injected into the reference channel. After an association stage of 900 s, the surface of the immunosensor was exposed to 10 mM PBS (pH 7.4) for 100 s, followed by 10 mM NaOH/0.5% SDS solution for 300 s, and finally 10 mM PBS (pH 7.4) in order to restore the baseline. The difference between the measurement and the reference channels was used to estimate the SPR signal generated during the non-specific sorption of SAV-QDs.

2.8. Explant preparation and detection of COMP by ELISA

After obtaining informed consent, human knee articular cartilage samples were collected from 3 different OA patients as tissues remaining after joint replacement surgeries (Vilnius Regional Bioethics Committee Approval No. 158200-14-741-257, Lithuania). The explants were excised from the surgically removed articular cartilage tissue, using biopsy punch, and transferred to 12 well plates. Explants were incubated in a chondrogenic medium consisting of high glucose (4.5 g/L) DMEM medium, 1% penicillin/streptomycin, 1% ITS, 0.35 mM L-proline, 1 μ M dexamethasone and 0.17 mM L-ascorbic acid 2-phosphate sesquimagnesium salt. The amount of medium was calculated according to explant weight (120 mg/3 mL). The medium was changed twice per week and the secretome was collected after 7 days of culture. Secretome samples were diluted 500 times with dilution buffer, and COMP concentration in the secretome was measured by a sandwich immunoassay using a human COMP ELISA kit. During the assay, secretome samples as well as standard samples were incubated in microplate wells pre-coated with capture antibodies against COMP. After 60 min incubation and washing, monoclonal anti-COMP_{17C10}-biot was added and incubated with captured COMP for 60 min. After another washing, SAV and horseradish peroxidase (HRP) conjugates (SAV-HRP) were added. After 30 min incubation and the last washing step, the remaining SAV-HRP were allowed to react with the 3,3',5,5'-tetramethylbenzidine solution. The reaction was stopped by the addition of stop solution and the absorbance of the resulting yellow product was measured using a Spectramax® i3x multi-mode microplate reader (Molecular Devices Ltd, Wokingham, United Kingdom). A calibration curve was constructed by plotting absorbance values against concentrations of the standard samples, and the unknown concentration of COMP in the secretome was determined using this calibration curve.

2.9. Detection of COMP in human knee articular cartilage secretome using the developed SPR immunosensor based on dual sandwich-type detection format

Secretomes of human knee articular cartilage explants with known COMP concentration of 105.59 (Patient P1), 151.02 (Patient P2) and 275.27 nM (Patient P3) were used for analysis. These secretomes were diluted 1000000-fold with 10 mM PBS (pH 7.4) to reach COMP concentrations of 105.59 (Patient P1), 151.02 (Patient P2) and 275.27 fM (Patient P1). The principle of COMP detection in secretomes was the same as described above for standard COMP samples. Firstly, Au/11-MUA–anti-COMP_{16F12} was incubated with diluted secretome, after interaction between anti-COMP_{16F12} and COMP, Au/11-MUA–anti-COMP_{16F12}/COMP was exposed to anti-COMP_{17C10}-biot, and finally Au/11-MUA–anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot was exposed to SAV–QDs. Pure PBS was injected into the reference channel during all analysis steps. The difference between the measurement and the reference channels was used to estimate the SPR signal generated during the anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot and SAV–QDs interaction.

2.10. Calculations

The surface concentration of immobilized capture anti-COMP_{16F12} antibodies was calculated considering that a change in SPR angle of 120 m° corresponds to 1 ng/mm² of surface bound biomolecules. The regeneration efficiency was evaluated by dividing the SPR signal recorded before the interaction (11-MUA–anti-COMP_{16F12} and COMP, 11-MUA–anti-COMP_{16F12}/COMP and anti-COMP_{17C10} or anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot and SAV–QDs) by the SPR signal recorded after restoring the baseline in 10 mM PBS (pH 7.4) after treating the immunosensor surface with regeneration solution and multiplying the obtained value by 100%. The SPR signal generated during interaction of 11-MUA–anti-COMP_{16F12} and COMP, 11-MUA–anti-COMP_{16F12}/COMP and anti-COMP_{17C10} or anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot and SAV–QDs at steady-state conditions (equilibrium angle) was calculated by approximating the results obtained during the association phase according to a 4-variable double rectangular hyperbola equation $y = ax/(b + x) + cx/(d + x)$, where sum of the variables a and c corresponds to the equilibrium angle, mo. LOD and LOQ were estimated as the concentration of COMP that gives an SPR signal equal to 3 or 10 standard deviations of the baseline noise, respectively. Experimental data were presented as the mean value of three independent measurements with error bars.

3. RESULTS AND DISCUSSION

This study describes the development and investigation of an SPR immunosensor for the ultra-sensitive quantitative determination of a COMP biomarker. Capture anti-COMP_{16F12} antibodies were immobilized on an 11-MUA SAM-coated SPR sensor disk using EDC and NHS coupling chemistry, which results a strong amide bond formation between the carboxyl groups of 11-MUA and the primary amino groups of antibodies. A dual sandwich-type signal amplification strategy using detection anti-COMP_{17C10-biot} antibodies and SAV-QDs was applied in the development of this immunosensor. According to it, in the first recognition step, immobilized capture anti-COMP_{16F12} antibodies and injected COMP were combined into an anti-COMP_{16F12} and COMP immune complex, which interacted with detection anti-COMP_{17C10-biot} antibodies in the second recognition step and finally, in the third recognition step, formed anti-COMP_{16F12}/COMP/anti-COMP_{17C10-biot} complex interacted with SAV-QD. Since the concentration of COMP controls the amount of bound detection anti-COMP_{17C10-biot} antibodies, and therefore the amount of SAV-QDs, the SPR signal generated by the binding of both anti-COMP_{17C10-biot} and SAV-QDs is proportional to the concentration of COMP. Due to the significantly higher mass compared to COMP and anti-COMP_{17C10-biot}, the binding of SAV-QDs through biotin-streptavidin interaction results in a much larger change in refractive index, leading to a significant increase in the SPR signal and an improvement in the sensitivity of the SPR immunosensor. An illustration of the immobilization of capture anti-COMP_{16F12} antibodies and the working principle of the developed SPR immunosensor is shown in Fig. 1.

3.1. Optimisation of anti-COMP_{16F12} concentration

The optimum concentration of capture anti-COMP_{16F12} antibodies to be immobilized was first evaluated. This is important in order to create optimal conditions for analyte quantification. A carbodiimide coupling chemistry based on the activation of surface carboxyl functional groups with a mixture of EDC and NHS was chosen for immobilization. For this, the gold surface of the SPR sensor disc was coated with 11-MUA SAM, which exhibits low non-specific adsorption (Stigter et al., 2005). The chosen covalent method of immobilization results a strong amide bond formation between the carboxyl groups of 11-MUA and the primary amino groups of antibodies. Although antibodies covalently linked to the surface via amino groups are oriented randomly, EDC/NHS coupling is a very simple and easy-to-perform process that does not require additional chemical modification of antibodies, and is therefore often used in the development of SPR and other immunosensors. The optimal concentration of capture antibodies was evaluated for immobilization

using 138.9, 277.8 and 416.7 nM anti-COMP_{16F12} solutions prepared in 10 mM NaAc buffer (pH 4.5) and observing the interaction of immobilized antibodies with COMP. The pH value of 4.5 of NaAc buffer was chosen based on the antibody pI and 11-MUA SAM pKa values. Since the pI of mouse IgG antibodies is between 6.4 and 7.6 (Danielsson et al., 1988) and the pKa of 11-MUA SAM as determined by potentiostatic infrared titration is 4.3 (Luque et al., 2014), in a medium with a pH of 4.5, anti-COMP_{16F12} has a positive charge, while remaining of non-activated carboxyl groups of 11-MUR SAM have a negative charge. Due to the electrostatic interaction, the antibody molecules are concentrated near the surface of the SPR sensor disk, and as a result, a larger amount of them is immobilized. As it can be seen from Fig. 2A, the surface concentration of immobilized anti-COMP_{16F12} increases with the concentration of the antibody solution. When 138.9, 277.8, and 416.7 nM antibody solutions were used, the calculated surface concentration of immobilized anti-COMP_{16F12} was 2.89 ± 0.11 , 4.98 ± 0.14 , and 5.87 ± 0.23 ng/mm², respectively. Thus, an increase in the concentration of the antibody solution from 277.8 to 416.7 nM no longer caused such a significant increase in surface concentration as an increase in the concentration of the antibody solution from 138.9 to 277.8 nM. The same tendency was observed when the interaction of immobilized antibodies with COMP was studied. The calculated SPR signals induced by 19.99 nM COMP were 19.45 ± 2.13 , 48.03 ± 2.58 and 53.85 ± 2.67 mo when 138.9, 277.8 and 416.7 nM antibody solutions were used for immobilization, respectively (Fig. 2 B). A similar effect of the surface concentration of immobilized antibodies on the SPR signal has been observed for other immunosensors and can be explained by the limited number of binding sites on the surface, which causes saturation of the signal generated by the immunosensor (Pawula et al., 2016). Taking into account the results of this experiment, a concentration of 277.8 nM anti-COMP_{16F12} was chosen as the optimal concentration for the immobilization because it was the best compromise between the magnitude of the immunosensor response and the cost-effective use of antibodies.

3.2. Optimisation of immunosensor surface regeneration conditions

Regeneration of the immunosensor surface between measurements is a necessary condition for accurate and repeatable analysis, as well as the operational stability of the immunosensor. During the regeneration, the ligand-analyte complex must be broken down, and the activity of the immobilized ligand must remain unchanged, it must not be inactivated or denatured, so the regeneration must be performed under as mild conditions as possible. If the regeneration is incomplete, the amount of free ligand on the surface of the immunosensor decreases and as a result a lower SPR signal can be recorded at the same analyte concentration. In order to avoid analytical errors, it is very important to

choose a suitable solution for regeneration that ideally provides 100% or as high regeneration efficiency as possible (Kausaite-Minkstimiene et al., 2022). Four commonly used regeneration solutions were used to regenerate the Au/11-MUA–anti-COMP_{16F12} surface: 10 mM glycine/HCl (pH 2.0), 10 mM glycine/HCl (pH 1.0), 10 mM NaOH/0.5% SDS and 25 mM NaOH/0.5% SDS. After immunoreaction of immobilized anti-COMP_{16F12} with COMP and subsequent dissociation of the immune complex in pure PBS, the surface of the immunosensor was exposed to regeneration solution for 300 s. From the obtained experimental data shown in Fig. 2C, it can be seen that significantly better regeneration efficiency was achieved with solutions consisting of 10 mM NaOH and 0.5% SDS and 25 mM NaOH and 0.5% SDS. The regeneration efficiency of these solutions was 98.97 ± 2.04 and $98.29 \pm 2.37\%$, respectively. Since the regeneration efficiency of these solutions was very similar, 10 mM NaOH/0.5% SDS was chosen for regeneration because of its lower aggressiveness that could potentially lead to inactivation of immobilized anti-COMP_{16F12} during repeated regenerations of the immunosensor surface. The optimal duration of regeneration was determined by exposing the immunosensor surface to 10 mM NaOH/0.5% SDS solution for different time intervals: 100, 200, 300, 400 and 500 s. The regeneration efficiency increased with the increase in the regeneration duration up to 200 s ($99.44 \pm 2.29\%$), and after this duration the regeneration efficiency did not improve and was within the error limits, as shown in Fig. 2D. Considering this and the possible inactivation of the immobilized anti-COMP_{16F12} during the repeated regeneration of the immunosensor surface, the regeneration duration of 200 s was chosen as optimal for the Au/11-MUA–anti-COMP_{16F12} surface.

3.3. Immunosensor based on direct detection format

The direct SPR immunoassay format, where the analytical signal is recorded during the interaction of the immobilized ligand with the analyte in solution without the use of any labels, is the simplest and least time- and reagent-intensive immunoassay format, and is therefore the first choice for the development of SPR immunosensors (Ramanaviciene et al., 2022). The ability of the immunosensor to directly detect and quantify COMP was investigated by monitoring the interaction of immobilized anti-COMP_{16F12} with COMP in solutions of different concentrations (0.50 – 20.16 nM). Concentration-dependent real-time sensorgrams recorded during the direct interaction of COMP with Au/11-MUA–anti-COMP_{16F12} are shown in Fig. 3A. For each sensogram, the SPR signal under steady-state conditions (equilibrium angle) was calculated and plotted as a function of COMP concentration, which is shown in Fig. 3B. As can be seen, the relationship between the equilibrium angle and COMP concentration was linear ($R^2 = 0.9994$). The calculated LOD and LOQ were 0.38

and 1.28 nM, respectively. Although somewhat different data are reported in the literature, the COMP levels in biological fluids are relatively very low. For example, Fawzy et al. (2011) reported that the serum COMP concentration in OA patients ranging from 0.97 to 2.65 $\mu\text{g/mL}$ (11.77 – 32.16 nM), with a mean of $1.25 \pm 0.37 \mu\text{g/mL}$ (15.17 ± 4.49 nM). According to Verma and Dalal (2013) study, serum COMP concentration ranging from 125.03 to 4209.75 ng/ml (1.52 – 51.09 nM) (median 1117.21 ng/ml (13.56 nM)) in knee OA patients and from 118 to 589 ng/ml (1.43 – 7.15 nM) (median 338.62 ng/ml (4.11 nM)) in control subjects. Whereas the serum concentration of COMP reported by Kraus et al. (2017) ranging from 403 to 5797 ng/ml (4.89 – 70.35 nM), with a mean of 1016.12 ng/ml (12.33 nM) and a median of 898.0 ng/ml (10.90 nM).

In addition, it must be considered that due to matrix effects caused by high molecular weight substances present in all biological fluids, samples must be diluted tenfold or more with a buffer prior to analysis. Therefore, the concentration of the analyte becomes significantly lower than in the original sample (Treviño et al., 2009). Consequently, in order to be appropriate for the analysis, the immunosensor should be able to quantify sub-nanomolar concentrations. Meanwhile, the estimated LOD and LOQ values indicate that the immunosensor based on the direct detection format is not sensitive enough and is therefore not appropriate for quantification of COMP in biological fluids.

3.4. Immunosensor based on conventional sandwich-type detection format

Since the magnitude of the immunosensor signal generated by the direct interaction of anti-COMP_{16F12} with COMP was insufficient to quantify the levels of COMP in biological samples, a conventional sandwich-type detection format was used for COMP quantification. For this purpose, detection anti-COMP_{17C10} antibodies were used in addition to immobilized capture anti-COMP_{16F12} antibodies. In the sandwich immunoassay, during the first recognition step, anti-COMP_{16F12}, immobilized on the SPR sensor disc, specifically interacts with the epitope of the COMP molecule and an antibody-antigen complex is formed. In the second recognition step, the detection anti-COMP_{17C10} antibody specifically interacts with another epitope of the COMP molecule of the formed anti-COMP_{16F12}/COMP immune complex. Since the concentration of COMP controls the amount of bound anti-COMP_{17C10}, the SPR signal generated during the interaction of anti-COMP_{17C10} with the anti-COMP_{16F12}/COMP immune complex is proportional to the concentration of COMP. Since the change in refractive index is proportional to the molecular weight of the molecule that binds to the immunosensor surface, the larger molecules change the refractive index more than the smaller, the interaction of anti-COMP_{17C10} (~150 kDa) with a twice higher molecular weight compared to COMP

(82.4 kDa) generates a higher SPR signal leading to an increased sensitivity of the immunosensor. In addition, the use of two antibodies that recognize different epitopes of the analyte molecule increases the accuracy of the analysis due to increased specificity (Lee et al., 2018).

COMP concentration-dependent real-time sensorgrams recorded during the interaction of anti-COMP_{17C10} with Au/11-MUA-anti-COMP_{16F12}/COMP are shown in Fig. 3C. 0.50, 1.26, 2.52, 5.04, 10.08 and 20.16 nM COMP and 100.19 nM anti-COMP_{17C10} solutions were used for the study. As in the case of the direct detection format, a linear relationship between equilibrium angle and concentration with $R^2 = 0.9997$ was valid over the range of COMP concentrations tested. The LOD and LOQ were found to be 0.063 and 0.21 nM, respectively (Fig. 3D). Compared to the direct detection format, the LOD and LOQ were about 6 times lower. Therefore, it can be concluded that the use of the anti-COMP_{17C10}, due to their higher molecular weight compared to COMP, allowed to improve the sensitivity of the immunosensor and it became suitable for the detection of lower concentrations of COMP. However, taking into account above mentioned COMP concentrations in biological samples and the need to dilute the sample, conventional sandwich-type detection format also does not provide the required sensitivity of analysis and is therefore not appropriate for quantification of COMP in biological fluids. For example, if serum COMP concentration is 1.43 – 7.15 nM (Verma and Dalal, 2013), then after tenfold dilution concentration becomes 0.14 – 0.72 nM, while calculated LOD is only 0.21 nM.

3.5. Immunosensor based on dual sandwich-type detection format

In order to further increase the sensitivity of the immunosensor, a dual sandwich-type signal amplification strategy using detection anti-COMP_{17C10}-biot antibodies and SAV-QDs was applied. According to it, during the first recognition step, immobilized capture anti-COMP_{16F12} antibodies specifically interacts with COMP molecules and form an anti-COMP_{16F12}/COMP immune complex, which interacted with detection anti-COMP_{17C10}-biot antibodies during the second recognition step. Finally, during the third recognition step, formed anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot complex interacted with SAV-QD. Since the concentration of COMP controls the amount of bound detection anti-COMP_{17C10}-biot, and therefore the amount of SAV-QDs, the SPR signal generated during third recognition step is proportional to the concentration of COMP. The SAV-QDs used in this study were composed of a CdSe nanocrystal coated with an additional ZnS shell and a polymer shell directly coupled to streptavidin. According to the manufacturer, SAV-QDs are ~15 – 20 nm in size, thus larger than any macromolecule or protein. Therefore, it was expected that SAV-QDs binding through biotin-

streptavidin interaction would lead to a very large change in refractive index due to the very large mass of SAV-QDs, which would significantly increase the SPR signal and improve the sensitivity of the immunosensor.

After it was assumed that the use of SAV-QDs would greatly improve the sensitivity of the immunosensor, the next step was to choose the most suitable concentration of anti-COMP_{17C10}-biot and SAV-QDs. The concentration of anti-COMP_{17C10}-biot was chosen based on literature data (Eletxigerra et al., 2016) and considering that these antibodies are the most expensive material required for the analysis and thus a trade-off between cost and performance is required. Therefore, a concentration of 55.37 nM anti-COMP_{17C10}-biot was chosen for the further studies. Meanwhile, the concentration of SAV-QDs was optimized considering the possibility of their non-specific sorption. The studies of SAV-QDs non-specific sorption were performed by observing their sorption on Au/11-MUA-anti-COMP_{16F12} surface. Solutions of 10.0, 20.0 and 30.0 nM SAV-QDs. As can be seen from the real-time sensorgrams recorded during the studies of non-specific sorption of SAV-QDs on Au/11-MUA-anti-COMP_{16F12} surface and the dependence of the equilibrium angle on SAV-QDs concentration shown in Fig. S1, the highest SPR signal of 85.54 m° was observed at the highest SAV-QDs concentration studied. However, it can also be seen that with increasing concentration of SAV-QDs, more of them remain on Au/11-MUA-anti-COMP_{16F12} after surface regeneration. This clearly illustrates that as the concentration of SAV-QDs increases, the non-specific sorption of SAV-QDs on the sensor surface also increases. Based on these results, a concentration of 10.00 nM anti-SAV-QDs was selected for signal amplification of the immunosensor.

Fig. 4A shows COMP concentration-dependent real-time sensorgrams recorded during the interaction of SAV-QDs with Au/11-MUA-anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot. Standard solutions of eight different concentrations of COMP (0.0, 2.80, 8.40, 25.21, 75.62, 226.85, 680.54 and 2242.12 fM) were tested. As can be seen from the sensorgrams and the dependence of the equilibrium angle on COMP concentration shown in Fig. 4B, the SPR signal increased with increasing COMP concentration over the entire concentration range studied. A linear relationship between COMP concentration and equilibrium angle with $R^2 = 0.9999$ was observed in the concentration range from 2.80 to 680.54 fM (Fig. 4C). The LOD and LOQ were found to be 0.15 and 0.50 fM, respectively. Compared to the conventional sandwich-type detection format, the LOD and LOQ were drastically reduced, demonstrating the high efficiency of SAV-QDs in SPR signal amplification. It is clear that SAV-QDs generate much higher SPR signal than antibodies due to their significantly higher mass. In comparison, the assay range of the standard ELISA kit for human COMP is 0.085–20 ng/mL (1.03 – 242.72 pM). Thus, the SPR immunosensor based on dual sandwich-type detection format developed

in this work allows the quantitative determination of COMP at significantly lower concentrations. On the other hand, taking into account COMP concentrations in biological samples and the required level of dilution, the proposed signal amplification strategy would be more suitable for quantification of analytes existing at much lower concentrations than COMP.

To our knowledge, only a few SPR immunosensors using QDs as high-mass signal-enhancing labels have been published so far. One of them, developed by Anderson et al. (2013), was intended for the detection of ricin. Analytical characteristics of the immunosensor are not provided in the paper, but QDs were demonstrated as very effective reporter elements in SPR sandwich-type detection format, providing more sensitive detection with a signal enhancement of about 10-fold.

Another SPR immunosensor, developed by Wang et al. (2016), was intended for the quantitative detection of α -fetoprotein, carcinoembryonic antigen, and cytokeratin fragment 21–1 in clinical samples. The developed SPR immunosensor was able to detect these analytes in the concentration range of 1 – 1000 ng/mL with LOD of 0.1 ng/mL. Considering the molecular weight of human α -fetoprotein (67.50 kDa (Yachnin et al., 1977)) and converting the LOD to molar concentration, the LOD is 1.48 pM. Thus, it can be stated that the LOD of the SPR immunosensor developed in the present work is significantly lower than that of the SPR immunosensor developed by Wang et al. (2016). A comparison of the analytical parameters of the developed immunosensor and some previously reported SPR immunosensors utilizing various nanomaterials as high-mass signal-enhancing labels is presented in Table 1.

A reliable immunosensor should have good reproducibility and repeatability of the analytical signal. These analytical characteristics directly depend on the choice of antibody immobilization method, loss of antibody activity and other factors. Therefore, the repeatability and reproducibility of the developed immunosensor based on dual sandwich-type detection format were also investigated. During repeatability studies, three real-time sensograms of SAV–QDs interaction with Au/11-MUA–anti-COMP_{16F12}/COMP/anti-COMP_{17C10-biot} were recorded one after the other at the same day using the same Au/11-MUA–anti-COMP_{16F12} immunosensor surface and the same concentration of COMP, anti-COMP_{17C10-biot} and SAV–QDs (75.62 fM, 55.37 nM and 10.00 nM, respectively). The estimated standard deviation (STDEV) and RSD were 4.89 and 8.05%, respectively (Table S1). In order to perform reproducibility studies, three Au/11-MUA–anti-COMP_{16F12} immunosensor surfaces were prepared under the same conditions. Then, using the same concentrations of COMP, anti-COMP_{17C10-biot} and SAV–QDs (75.62 fM, 55.37 nM and 10.00 nM, respectively), a real-time sensogram during SAV–QDs interaction with Au/11-MUA–anti-COMP_{16F12}/COMP/anti-COMP_{17C10-biot} was recorded with each of them. The estimated STDEV and RSD were 5.87 and 9.88%, respectively (Table S1).

Higher inaccuracy between different Au/11-MUA–anti-COMP_{16F12} immunosensor surfaces compared to the same one may be due to slightly varying surface concentration of immobilized anti-COMP_{16F12} and loss of antibody activity during sensor surface preparation and analysis. In summary, the developed immunosensor showed reasonably good repeatability and reproducibility of the analytical signal, regardless of analytical errors. It can be stated that it is accurate enough to be used for analysis.

The operational stability of the developed immunosensor was also studied. In this study, the variation in the magnitude of the analytical signal generated by the immunosensor (equilibrium angle) during the interaction of SAV–QD with Au/11-MUA–anti-COMP_{16F12}/COMP/anti-COMP_{17C10-biot} was monitored over a period of 13 days. The same immunosensor and the same concentrations of COMP, anti-COMP_{17C10-biot} and SAV–QDs (75.62 fM, 55.37 nM and 10.00 nM, respectively) were used in the study. Between measurements, the immunosensor was kept in a controlled environment (10 mM PBS (pH 7.4), 20 °C). As can be seen from the data presented in Fig. S2, after 13 days, the size of the equilibrium angle decreased by only 2.14% compared to that recorded on the first day of the study. It is likely that such high operational stability of the developed immunosensor could be achieved thanks to the applied covalent method of antibody immobilization. Despite the random orientation, the anti-COMP_{16F12} are connected to the surface of the immunosensor by a strong amide bond, which prevents desorption of the antibodies from the surface during repeated measurements and washing the immunosensor between measurements with a buffer solution. Covalently immobilized proteins are also more resistant to environmental changes (Rezvanian et al., 2018), which may be the cause of loss of activity during storage. The results of the study show that the loss of immobilized anti-COMP_{16F12} activity during regeneration was also avoided. Thus, according to the results obtained, it can be concluded that the developed immunosensor has excellent operational stability and is suitable for long-term analysis.

The suitability of the developed immunosensor for the analysis of real samples was tested by determining the amount of COMP in the secretome of explants from human knee articular cartilage samples obtained from 3 different OA patients. First, to find out the actual concentration, COMP concentrations in the secretome were measured by a standard sandwich immunoassay, using a human COMP ELISA kit and the following concentrations were found: 105.59 (Patient P1), 151.02 (Patient P2), and 275.27 nM (Patient P3). These secretomes were then diluted 100000-fold in 10 mM PBS (pH 7.4) to obtain COMP concentrations of 105.59 (Patient P1), 151.02 (Patient P2) and 275.27 fM (Patient P3). Analysis of diluted secretomes was performed using the same immunosensor and the same concentration of anti-COMP_{17C10-biot} and SAV–QDs (55.37 and 10.00 nM, respectively). From

the real-time sensorgrams recorded during the interaction of SAV-QDs with Au/11-MUA-anti-COMP_{16F12}/COMP/anti-COMP_{17C10-biot}, the equilibrium angle was calculated for each sample. The concentration of COMP in the diluted secretomes was determined from a linear equation derived for a calibration curve constructed from data obtained by analysing standard COMP solutions in the range 2.80 – 680.54 fM. COMP concentration in secretomes was calculated by considering the dilution. As can be seen from the data presented in Table 2, the analysis error was less than 5%. This shows that the developed immunosensor has good accuracy and possibility of applying it to COMP analysis not only in the secretomes of human knee articular cartilage samples, but also in other biological samples.

4. CONCLUSIONS

We developed a novel SPR immunosensor for the ultra-sensitive quantitative determination of COMP, an exploratory biomarker of joint tissue ECM turnover in arthritic diseases. A dual sandwich-type signal amplification strategy using anti-COMP_{17C10-biot} and SAV-QDs enabled a drastic increase in the sensitivity of the analysis. The increase in sensitivity was due to the binding of very high-mass SAV-QDs via biotin-streptavidin interaction to the surface of the immunosensor, which resulted in a very high change in refractive index and, consequently, a very high SPR signal. The results showed that the developed immunosensor was able to detect concentrations of COMP in a range from 2.80 to 680.54 fM with the LOD and LOQ of 0.15 and 0.50 fM, respectively. These are significantly lower concentrations than can be detected by a standard sandwich immunoassay using a human COMP ELISA kit. Furthermore, to the best of our knowledge, in this work we developed the most sensitive SPR immunosensor using QDs as high-mass signal-enhancing labels reported so far. It also showed good repeatability and reproducibility as well as excellent operational stability. The analysis of secretomes of human knee articular cartilage samples revealed that the immunosensor has good accuracy and is suitable for the analysis of real samples. The dual sandwich-type signal amplification strategy used in this work can be very useful in the development of SPR immunosensors for the quantitative determination of other analytes that exist in biological fluids at extremely low concentrations. Thus, the proposed signal amplification strategy could in principle be used for the quantification of such low molecular weight analytes that cannot be detected either by conventional sandwich-type detection format or other methods of indirect detection format. Such SPR immunosensors would be potential tools for highly sensitive clinical analysis and promising alternatives to traditional bioanalytical methods. In addition, if necessary, the analysis could be combined with quantum dot-linked immunosorbent assay. Current research in our group is directed

towards the development of new highly sensitive SPR immunosensors for the quantification of other clinically relevant analytes using various high-mass nanomaterials as signal-enhancing labels.

CRedit authorship contribution statement

Asta Kausaite-Minkstimiene: Conceptualization, Methodology, Data curation, Visualization, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. Anton Popov: Investigation, Writing – original draft, Writing – review & editing. Ursule Kalvaityte: Investigation, Data curation, Writing – original draft, Writing – review & editing. Eiva Bernotiene: Resources, Project administration, Writing – original draft, Writing – review & editing. Ali Mobasher: Funding acquisition, Writing – original draft, Writing – review & editing. Almira Ramanaviciene: Supervision, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This research was funded by the European Regional Development Fund through the Research Council of Lithuania according to the Programme “Attracting Foreign Researchers for Research Implementation”, Grant No. 01.2.2-LMT-K-718-02-0022.

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Tables

Table 1. Comparison of the analytical parameters of the developed immunosensor and previously reported SPR immunosensors utilizing nanomaterials as high-mass signal-enhancing labels.

Analyte	High-mass enhancing label	signal- Linear range	LOD	Ref.
COMP	SAv-QDs	2.80– 680.54 fM	0.15 fM	this work
Tau protein	MW-CNT	–	125 pM	Lisi et al. (2017)
SEG	SiNPs	–	10 pM	Belen et al. (2021)
17 β -estradiol	MNPs	1.95– 2000 ng/mL	0.81 ng/mL	Jia et al. (2018)
CD5	mAuNPs	0.05–99.26 pM	8.31 fM	Kausaite-Minkstimiene et al. (2022)
cTnT	AuNPs	0.5–40 ng/mL	0.5 ng/mL	Pawula et al. (2016)
ErbB2	SAv-AuNPs	–	180 pg/mL	Eletxigerra et al. (2016)
gIgG	AgNPs	–	6.66 nM	Paul et al. (2011)
PSA-ACT	QDs	–	0.1 ng/mL	Malic et al. (2011)
AFP, CYFRA 21-1	CEA, QDs	1–1000 ng/mL	0.1 ng/mL	Wang et al. (2016)

MW-CNT – multi-walled carbon nanotubes; SEG – staphylococcal enterotoxin G; SiNPs – silica nanoparticles; MNPs – magnetic nanoparticles; mAuNPs – gold-coated magnetic nanoparticles; cTnT – cardiac troponin T; AuNPs – gold nanoparticles; ErbB2 – epidermal growth receptor factor 2; SAv-AuNPs – SAv-conjugated AuNPs; gIgG – goat immunoglobulin G; AgNPs – silver nanoparticles; PSA-ACT – prostate specific antigen- α 1-antichymotrypsin complex; AFP – α -fetoprotein; CEA – carcinoembryonic antigen; CYFRA 21-1 – cytokeratin fragment 21-1.

Table 2. Data obtained from the determination of COMP in secretomes of human knee joint articular cartilage samples.

Sample	Stated concentration ELISA, nM	by Equilibrium angle, m^o	Detected concentration diluted sample, fM	Detected in concentration sample, nM	in Error, %
Patient P1	105.59	67.88	109.95	109.95	+4.13
Patient P2	151.02	75.70	149.05	149.05	-1.30
Patient P3	275.27	98.75	264.30	264.30	-3.99

Figure legends

Fig. 1. An illustration of the immobilization of capture anti-COMP_{16F12} antibodies and the working principle of the developed SPR immunosensor for COMP biomarker quantification.

Fig. 2. Effect of the concentration of anti-COMP_{16F12} antibodies on their surface concentration (A) and the magnitude of the immunosensor response to COMP (B). Dependence of regeneration efficiency on used regeneration solution (C) and regeneration duration (D). Experimental conditions: 277.78 nM anti-COMP_{16F12} in 10 mM NaAc buffer (pH 4.5) (B, C), 19.99 nM COMP in 10 mM PBS (pH 7.4), 300 s duration of regeneration (A, B). Error bars represent the standard deviation of three replicates (n = 3).

Fig. 3. SPR sensograms recorded during direct interaction of COMP with Au/11-MUA-anti-COMP_{16F12} (A) and calibration curve (B). SPR sensograms recorded during interaction of anti-COMP_{17C10} with Au/11-MUA-anti-COMP_{16F12}/COMP (C) and calibration curve (D). Experimental conditions: 277.78 nM anti-COMP_{16F12} in 10 mM NaAc buffer (pH 4.5), 0.50, 1.26, 2.52, 5.04, 10.08 and 20.16 nM COMP in 10 mM PBS (pH 7.4), 100.19 nM anti-COMP_{17C10} in 10 mM PBS (pH 7.4), 10 mM NaOH/0.5% SDS regeneration solution, 200 s duration of regeneration. Error bars represent the standard deviation of three replicates (n = 3).

Fig. 4. SPR sensograms recorded during interaction of SAV-QDs with Au/11-MUA-anti-COMP_{16F12}/COMP/anti-COMP_{17C10}-biot (A), dependence of the equilibrium angle on COMP concentration (B) and calibration curve (C). Experimental conditions: 277.78 nM anti-COMP_{16F12} in 10 mM NaAc buffer (pH 4.5), 0.0, 2.80, 8.40, 25.21, 75.62, 226.85, 680.54 and 2242.12 fM COMP in 10 mM PBS (pH 7.4), 55.37 nM anti-COMP_{17C10}-biot in 10 mM PBS (pH 7.4), 10.00 nM SAV-QDs in 10 mM PBS (pH 7.4), 10 mM NaOH/0.5% SDS regeneration solution, 200 s duration of regeneration. Error bars represent the standard deviation of three replicates (n = 3).







