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Murine double minute 2 (MDM2) is an oncoprotein, mediating the degradation of tumor suppressor p53 protein. The physiological levels of MDM2 protein are closely related to malignant transformation and tumor growth. In this work, simultaneous and label-free determination of free and p53-bound MDM2 proteins from sarcoma tissue extracts was conducted using a dual-channel surface plasmon resonance (SPR) instrument. Free MDM2 protein was measured in one fluidic channel covered with consensus double-stranded (ds)-DNA/p53 conjugate, while MDM2 bound to p53 was captured by the consensus ds-DNA immobilized onto the other channel. To achieve higher sensitivity and to confirm specificity, an MDM2-specific monoclonal antibody (2A10) was used to recognize both the free and p53-bound MDM2 proteins. The resultant method afforded a detection limit of 0.55 pM of MDM2. The amenability of the method to the analysis of free and p53-bound MDM2 proteins was demonstrated for normal and sarcoma tissue extracts from the three patients. Our data reveal that both free and total MDM2 (free and bound forms combined) proteins from sarcoma tissue extracts are of much higher concentrations than those from normal tissue extracts and the p53-bound MDM2 protein only constitutes a small fraction of the total MDM2 concentration. In comparison with enzyme-linked immunosorbent assay (ELISA), the proposed method possesses higher sensitivity, is more cost-effective, and is capable of determining free and p53-bound MDM2 proteins in clinical samples.

Introduction

Murine double minute 2 (MDM2) is a key negative regulator of tumor suppressor p53 protein, inhibiting the activity of p53 protein and reducing the p53 concentration through the p53-MDM2 feedback loop.[1-4] The overexpression of MDM2 protein is considered as a common mechanism of cancer development and progression.[5-7] It has been documented that MDM2 protein is overexpressed in 7% of human tumor cells and 20% of tumor tissues.[8, 9] The actual physiological MDM2 levels in cancerous tissues could be even higher due to enhanced translation and gene translocation.[10] Monitoring of the abnormally high levels of MDM2 protein is of significance for cancer diagnosis and formulation of appropriate treatment modality.

Conventional methods for MDM2 protein assay include enzyme-linked immunosorbent assay (ELISA),[11] Western blotting,[12] immunofluorescence assay,[13] and immunohistochemistry assay.[14] ELISA is a standard quantitative method for the detection of total MDM2 protein (free and bound forms combined) in vitro, however, a typical ELISA requires two antibodies and does not differentiate free MDM2 from its complexed counterpart.[11, 15] Western blotting is capable of separating protein components according to their sizes, but is time-consuming, semi-quantitative, and involves a radioactive label.[12, 16] Recently, MDM2 assay for cancer staging has been performed via combination of a host-guest complex probe with catalytic activity and a signal amplification strategy to augment the electrochemical signals.[17] Elshafey et al. developed an ultrasensitive and label-free impedimetric immunosensor for assaying MDM2 in cancerous mouse brain tissue.[18] A target-specific fluorescent "light-up" probe for measuring cellular MDM2 protein and for drug screening has been reported.[19] Using single-molecule microarray, the levels of p53 and MDM2 proteins from crude cell lysates were measured.[20] Retout et al. reported a colorimetric assay for MDM2 oncoprotein based on peptide aptamer-functionalized gold nanoparticles.[8] However, none of the above methods are capable of simultaneously measuring free and bound MDM2 proteins. Moreover, they did not compare the MDM2 concentrations in the normal and cancerous tissues from the biopsies of the same patient.

Surface plasmon resonance (SPR) serves as a viable alternative for clinical use, which enables label-free and real-time monitoring of clinically related biomolecules with high sensitivity. For example, facile and sensitive analyses of a series of species, such as antibody, protein, enzyme, DNA, therapeutic drugs and other classes of molecules in human

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samples has been performed by SPR.[21] Previously we have used voltammetry[22] and SPR[23] to assay both wild-type and mutant p53 proteins in normal and cancer cell lysates. The high affinity of consensus ds-DNA to the DNA-binding domain of p53 protein has proven to be an efficient way for capturing the wild-type p53 protein.[22, 23] In this study, via the specific binding of p53 protein to MDM2 and consensus ds-DNA to the pre-formed p53/MDM2 complex, simultaneous SPR detection of both free and p53-bound MDM2 proteins in sarcoma tissue extracts was accomplished. The proposed method is sensitive and selective, providing a facile means for detecting a key oncoprotein in clinical samples.

Experimental

Materials

11-mercaptoundecanoic acid (MUA), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), ethanolamine hydrochloride (EA), KH_2PO_4, and K_2HPO_4 were acquired from Sigma (St. Louis, MO). Oligonucleotides of various sequences were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Immobilization of the consensus ds-DNA was achieved by anchoring the aminated oligonucleotide (5′-H_2N-$(CH_2)_6$-TTT TTA GAC ATG CCC AGA CAT GCC C-3′) on the MUA self-assembled monolayers (SAMs), followed by hybridization to its complementary strand (5′-GGG CAT GTC TGG GCA TGT CT-3′). The sequences of the non-consensus ds-DNA are 5′-H_2N-$(CH_2)_6$-TTT TTG TCG GCC GAG GTC GGC CGA G-3′ and 5′-CTC GGC CGA CCT CGG CCG AC-3′. Recombinant p53 protein was acquired from BD Biosciences Pharmingen (San Diego, CA). Recombinant human MDM2 protein, MDM2-specific monoclonal antibody (2A10), human epidermal growth factor receptor 2 (HER2), recombinant human MUC1 protein, IgG, and BSA were obtained from Abcam (Cambridge, MA). Carcino-embryonic antigen (CEA) was obtained from Xinyu Biotechnology Co., Ltd. (Shanghai, China). Other reagents were of analytical purity and used as received. All stock solutions were prepared daily with deionized water treated with a water purification system (Simplicity 185, Millipore Corp., Billerica, MA).

Instruments

The SPR measurements were conducted on a BI-SPR 3000 system (Biosensing Instrument Inc., Tempe, AZ). The light source of the instrument is diode lasers with an output of up to 1 mW of visible radiation at 670 nm. Phosphate-buffered saline (PBS, 10 mM phosphate, pH 7.4) containing 100 mM NaCl and 0.01% (v/v) Tween 20 was degassed via vacuum pumping for 30 min and used as the carrier solution. The recombinant MDM2 standards or tissue extracts were preloaded into a 200-μL sample loop and delivered to the flow cell by a syringe pump (Model KDS260, KD Scientific, Holliston, MA). The flow rate was 20 μL/min.

Procedures

Solution preparation

The aminated oligonucleotide was dissolved in PBS, and its complementary strand was prepared with PBS containing 5 mM $MgCl_2$. The solutions of MDM2 protein, p53 protein, and tissue extracts were prepared or diluted with PBS. MUA and EA were dissolved in ethyl alcohol and water, respectively. EDC/NHS solution was prepared by mixing 0.4 M EDC and 0.1 M NHS in water before the activation of MUA SAMs.

SPR chip modification

Gold films coated onto BK7 glass slides were purchased from Biosensing Instrument Inc. and annealed in a hydrogen flame. The gold films were immersed in 500 μM MUA solution for 24 h and the resultant SAMs were rinsed with ethanol, water, and dried under nitrogen. For DNA immobilization, 200 μL EDC/NHS solution was injected onto the sensor chip for activation of the carboxyl groups on MUA for 7.5 min, which was followed by attaching 2 μM of single-stranded oligonucleotide. EA (1 M) was used to block the unreacted sites and the incubation time was 5 min. The as-immobilized strand was hybridized to its complementary strand in a 2 μM solution for 30 min. Further injection of 5 nM p53 protein leads to formation of the ds-DNA/p53 conjugate.

SPR detection of MDM2 proteins in tissue extracts

The homogeneous tissue extracts were obtained from SunYat-Sen University Cancer Center (Guangzhou, China), approved by the Ethics Committee of SunYat-Sen University Cancer Center Health Authority. The collection and use of the tissue extracts followed the procedures that were in accordance with the ethical standards as formulated in the Helsinki Declaration.[24] MDM2 standards or the tissue extracts were injected onto the sensor chip, followed by injecting 1.5 nM MDM2-specific monoclonal antibody, 2A10, which was used to recognize both free and p53-bound MDM2 proteins and amplify the SPR signals.

ELISA assay of total MDM2 proteins in tissue extracts

The ELISA kit was purchased from USCN Life Science Inc. (Wuhan, China). Quantitative measurements of total MDM2 protein from the normal and sarcoma tissue extracts were performed on a 96-well plate reader (Tecan Trading AG, Switzerland).

Results and discussion

Principle of MDM2 assay

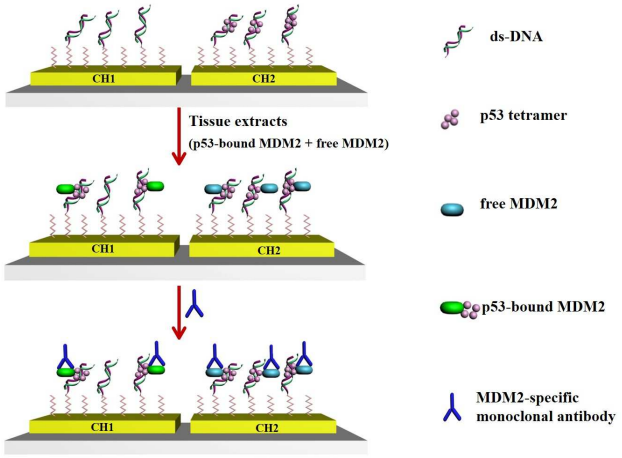

Figure 1. Schematic of the simultaneous SPR detection of p53-bound (fluidic channel 1, CH1) and free (fluidic channel 2, CH2) MDM2 proteins. The CH1 and CH2 were pre-immobilized with consensus ds-DNA and ds-DNA/p53 conjugate, respectively. Injections of normal and sarcoma tissue extracts result in the attachment of p53-bound and free MDM2 proteins in CH1 and CH2, respectively. The MDM2-specific monoclonal antibody, 2A10, was used to recognize both the free and p53-bound MDM2 proteins and amplify the SPR signals.

Figure 1 shows the schematic for simultaneous detection of free and p53-bound MDM2 proteins. The SAMs of consensus ds-DNA were first formed on MUA-covered gold chips via coupling of the amine-terminated single-stranded oligonucleotide, followed by hybridization with its complementary strand. The fluidic channel 1 (CH1) and 2 (CH2) were pre-immobilized with consensus ds-DNA and ds-DNA/p53 conjugate,[25] respectively. When the tissue extracts were flowed through the sensor chips, the p53-bound and free MDM2 proteins were respectively bound to CH1 and CH2. Thus, CH1 detects the p53-bound MDM2, while CH2 determines the free form of MDM2. The three hydrophobic residues (Phe19, Trp23, and Leu26) at the N-terminus of p53 protein are responsible for the formation of the p53-MDM2 complex.[26, 27] It has been documented that both free and bound forms of MDM2 protein could be recognized by MDM2-specific monoclonal antibodies in clinical samples.[28-30] An MDM2-specific monoclonal antibody, 2A10, was therefore injected onto the fluidic channels to recognize both the free and p53-bound MDM2 proteins. By examining the amplified SPR signals of CH1 and CH2, simultaneous and confirmatory determination of free and bound forms of MDM2 protein could be achieved.

SPR detection of MDM2 proteins

As shown in Figure 2, an injection of 0.5 nM MDM2 protein onto the fluidic channels pre-immobilized with the ds-

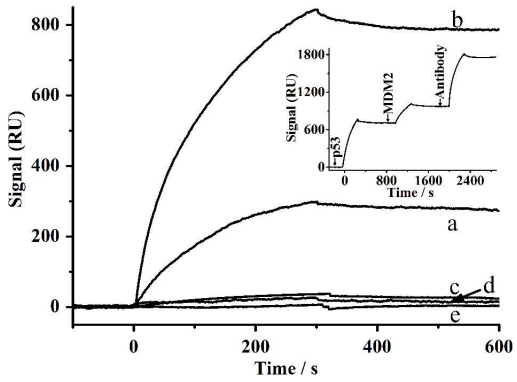

Figure 2. Background-subtracted SPR sensorgrams corresponding to the injection of 1.5 nM MDM2-specific monoclonal antibody (2A10) onto the fluidic channels covered with (b) consensus ds-DNA/p53/MDM2 protein, (c) consensus ds-DNA/MDM2 protein, (d) consensus ds-DNA/p53 protein, and (e) non-consensus ds-DNA/p53/MDM2 protein. The concentration of MDM2 protein injected was 0.5 nM. Curve a shows the SPR response upon injection of 0.5 nM MDM2 onto the sensor chip modified with consensus ds-DNA/p53 protein. The inset shows the continuous SPR sensorgram on the fluidic channels covered with consensus ds-DNA upon injection of 5 nM p53, 0.5 nM MDM2, and 1.5 nM 2A10. The flow rate was 20 μL/min.

DNA/p53 conjugate produced an SPR signal of 268.1 RU (curve a). Because the expression of MDM2 oncoprotein in biological tissue samples is usually at low pM levels,[17] an MDM2-specific monoclonal antibody (2A10) was used to amplify the signal. As shown in curve b, injection of the antibody not only augmented the signal to 777.8 RU (curve b), but also provided additional assurance about the specific p53/MDM2 interaction. The signal amplification stems from the fact that the antibody's molecular weight (150 kDa) is higher than MDM2 (55 kDa).[31] No binding signal was expected from curve c because without p53 the consensus ds-DNA cannot recognize MDM2. In the absence of MDM2, the injection of the MDM2 antibody also yielded no signals, indicating that non-specific adsorption of the antibody is negligible (curve d). We also did not detect any SPR signal after the MDM2 antibody was injected onto the fluidic channels covered with non-consensus ds-DNA/p53/MDM2 (curve e), suggesting that binding of p53 tetramers to the consensus ds-DNA is essential for our sensor.[22] To further illustrate these interactions which are in sequence, the continuous injections of p53, MDM2, and 2A10 onto the fluidic channels covered with consensus ds-DNA were performed (inset of Figure 2).

Specificity of MDM2 assay

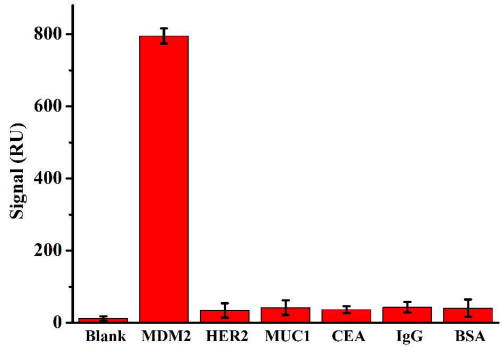

Figure 3. The specificity of MDM2 assay. The injection of MDM2, HER2, MUC1, CEA, IgG or BSA onto the fluidic channels covered with consensus ds-DNA/p53 protein was followed by the injection of 1.5 nM monoclonal antibody 2A10. The concentrations of MDM2, HER2, MUC1, CEA, IgG and BSA were all maintained at 0.5 nM. The blank column was obtained in the absence of MDM2. Other experimental conditions are the same as those in Figure 2.

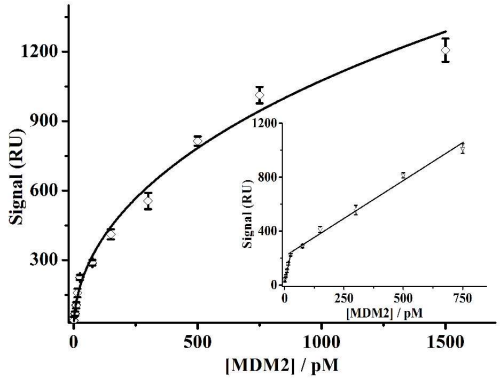

Figure 4. Dependence of the SPR signals on MDM2 concentrations. The exposure of the consensus ds-DNA/p53 to MDM2 was followed by the injection of 1.5 nM monoclonal anti-MDM2 antibody. The MDM2 concentrations are 1, 5, 10, 15, 25, 75, 150, 300, 500, 750, and 1500 pM. The inset shows the two linear portions of the calibration curve. The error bars are absolute errors deduced from at least three replicate measurements And the RSDs for all the concentrations were below 13 %.

To demonstrate the specificity of the method, other proteins, such as HER2, MUC1, IgG, CEA, and BSA were tested on the fluidic channels covered with consensus ds-DNA/p53 conjugate (Figure 3). The first four proteins are oncoproteins or proteins involved in the immune system, while BSA serves as a generic protein given that it is widely used as a protein for surface adsorption/blocking.[32, 33] On the basis that the SPR signals are all less than 5.5% of that produced by MDM2 protein, it is evident that other proteins did not exhibit appreciable non-specific adsorption or render cross-reactivity. That the injection of the MDM2 antibody in the absence of MDM2 did not produce any detectable SPR signal (blank) suggests that our assay is highly specific. Thus, our method can be a viable means for the determination of MDM2 protein in clinical samples.

Calibration curve of the MDM2 assay

The feasibility of the method for MDM2 quantification is shown in Figure 4. The SPR signals increased linearly with the concentrations of MDM2 from 1 to 25 pM and 25 pM to 750 pM and began to level off beyond 750 pM. The inset shows the two linear portions of the calibration curve and the linear regression equations are expressed as Signal (RU) = 44.06 + 10.09 [MDM2] (1–25 pM) and Signal (RU) = 203.8 + 1.168 [MDM2] (25–750 pM). The limit of detection was estimated to be 0.55 pM based on S/N=3,[34] lower than that achievable with the commercial ELISA kit (63 pg/mL or 1.15 pM).[35] Such a detection level is also four or five orders of magnitude lower than those by the colorimetric assay using gold nanoparticles functionalized with peptide aptamers (20 nM) [8] and SPR imaging microarray (72 nM).[36] As will be shown below in connection to our assays of extracts of cancerous tissues, only detection levels at pM or lower are clinically relevant. The calibration curve was used for the quantification of both free and p53-bound MDM2 proteins in real samples because either free or complexed MDM2 could be recognized by the monoclonal antibody.

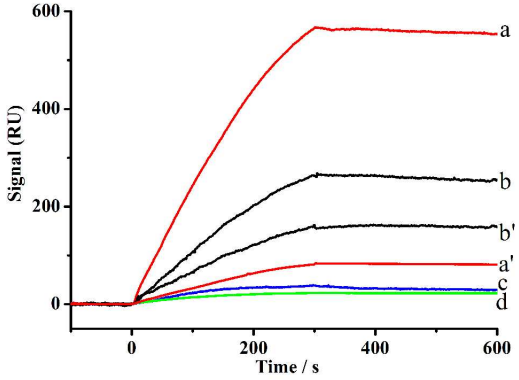

Figure 5. Background-subtracted SPR assay of free and p53-bound MDM2 proteins in 2.5-fold diluted human sarcoma tissue extract (curves a and a' for free and p53-bound MDM2 proteins, respectively) and normal tissue extract from the same sarcoma patient (curves b and b' for free and p53-bound MDM2 proteins, respectively). Obtained with a chip covered with non-consensus ds-DNA/p53 protein are curves c and d from the sarcoma tissue extract and normal tissue extract, respectively. In all the cases, 1.5 nM MDM2-specific monoclonal antibody was used to recognize both the free and p53-bound MDM2 proteins and amplify the SPR signals.

Table 1. Free and p53-bound MDM2 proteins in sarcoma and normal tissue extracts assayed by SPR and ELISA (n=3)

Clinical samples	Tissue extracts	SPR (nM)			ELISA (nM)
		Free MDM2	p53-bound MDM2	Total MDM2	Total MDM2
1	normal	0.043 ± 0.005	0.010 ± 0.003	0.053 ± 0.006	0.052 ± 0.018
	sarcoma	0.736 ± 0.012	0.007 ± 0.004	0.743 ± 0.012	0.763 ± 0.009
2	normal	0.048 ± 0.004	0.011 ± 0.003	0.059 ± 0.005	0.062 ± 0.004
	sarcoma	0.659 ± 0.024	0.009 ± 0.004	0.669 ± 0.024	0.698 ± 0.003
3	normal	0.236 ± 0.010	0.056 ± 0.005	0.292 ± 0.011	0.311 ± 0.013
	sarcoma	1.051 ± 0.014	0.047 ± 0.008	1.098 ± 0.016	1.151 ± 0.021

Detection of free and p53-bound MDM2 proteins in human sarcoma tissue extracts

The SPR sensorgrams for the detection of free and p53-bound MDM2 proteins in 2.5-fold diluted sarcoma tissue extract and normal tissue extract from the same sarcoma patient are shown in Figure 5. A large SPR signal of 550.9 RU corresponding to free MDM2 in the sarcoma tissue extract was observed (curve a). For the normal tissue extract, a smaller SPR signal of 248.9 RU was attained (curve b), suggesting that free MDM2 protein was overexpressed in this sarcoma tissue extract. This is consistent with the finding that the expression of MDM2 protein in tumors is higher than that in healthy tissues.[37, 38] Interestingly, the level of p53-bound MDM2 protein in sarcoma tissue extract (81.34 RU, curve a') is lower than that in normal tissue extract (170.1 RU, curve b'). In addition, for both normal and sarcoma tissue extracts, the amount of free MDM2 exceeds that of the complexed form. As evidenced by the negligible SPR signals upon injection of the sarcoma tissue extract and normal tissue extract over the sensor chips immobilized with non-consensus ds-DNA/p53 protein (curves c, d, respectively), our method is highly selective for assaying MDM2 in clinical samples.

To further demonstrate the clinical relevance, additional clinical samples were measured (Table 1). These samples were also analysed by ELISA in which the total MDM2 concentration was determined.[39] The concentration of total MDM2 protein measured by our method is in excellent agreement with that by ELISA. In comparison with those in normal tissue extracts, remarkably higher levels of free and total MDM2 proteins were obtained in the sarcoma tissue extracts (clinical samples 1 to 3). For example, the levels of free MDM2 protein in the assayed sarcoma tissue extracts are about 4.5- to 17-fold higher than those in the corresponding normal tissue extracts from the same sarcoma patients. It is worth noting that the sarcoma tissue extracts possessed similar levels of p53-bound MDM2 protein when compared to the corresponding normal tissue extracts, but the percentage of the bound MDM2 relative to total MDM2 from the sarcoma tissue extracts (0.9–4.3%) was much lower than that from the corresponding normal tissue extracts (~19%). It is therefore clear that MDM2 is overexpressed in the tumor tissues, which compromises the regulation of cell growth by p53,[40, 41] and reduces the p53 concentration that is critical for a cascade of events such as sensing of damaged DNA,[42] DNA repair,[43] and the initiation of apoptosis of cancerous cells.[1] To our knowledge, this is the first study quantifying the percentages of MDM2 bound by p53 with respect to the total MDM2 and demonstrating that the p53-bound MDM2 only constitutes a small fraction of the total MDM2 concentration. Our method obviates the need for enzyme-conjugated secondary antibody in ELISA. Such a method is helpful for clinicians and researchers in identifying the etiology of certain cancers.

Conclusions

Simultaneous SPR determination of free and p53-bound MDM2 proteins has been accomplished in fluidic channels pre-immobilized with consensus ds-DNA/p53 protein and consensus ds-DNA, respectively. An MDM2-specific monoclonal antibody (2A10) was used for specific recognition of free and p53-bound MDM2 protein, providing high sensitivity and specificity of the assay. The sensing protocol leads to a detection limit of MDM2 protein down to 0.55 pM, lower than those by the commercially available ELISA kit and the colorimetric assay using peptide aptamers-functionalized

gold nanoparticles. The method is advantageous over ELISA in that the levels of both free and p53-bound MDM2 proteins in sarcoma and normal tissue extracts could be determined. Remarkably higher levels of free MDM2 protein were obtained in the sarcoma tissue extracts than those in the normal tissue extracts and the p53-bound MDM2 protein only constituted a small fraction of the total MDM2 concentration. The proof-of-concept experiments demonstrate that SPR is simple, sensitive, selective, and cost-effective and can be used for potential cancer diagnoses in clinical settings.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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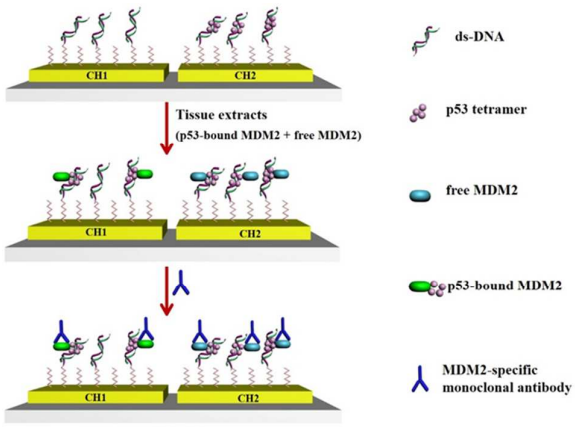
Graphical and textual abstract



Sensitive SPR determination of free and p53-bound MDM2 proteins from sarcoma tissue extracts was carried out in fluidic channels covered with consensus ds-DNA/p53 conjugate and the consensus ds-DNA, respectively.