SUPPLEMENTARY MATERIAL

Dual immunoplatform to assess senescence biomarkers TIMP-1 and GDF-15: Advancing in the understanding of colorectal cancer

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Experimental part

Apparatus and electrodes

Amperometric measurements were performed at room temperature using a CHI1030B potentiostat provided with the CHI1030B software. Single screen-printed carbon electrodes (SPCEs, DRP110), dual SPCEs (SPdCEs, DRP-X1110), and their connecting cables (DRP-CAC and DRP-BICAC, respectively) were purchased from Metrohm-DropSens. The SPCE and SPdCEs consisted of three-electrode cell configurations containing one circular ($\emptyset = 4$ mm) or two elliptical ($\emptyset = 2$ mm) carbon working electrode/s, respectively, a carbon auxiliary electrode, and an Ag *pseudo*-reference electrode. The electrodes were inserted in a homemade polymethylmethacrylate (PMMA) casing with either one or two embedded neodymium magnet/s (AIMAN GZ) to capture the modified magnetic beads (MBs) on the SPCE or SPdCEs working electrode/s. A 10 mL glass electrochemical cell was used for amperometric measurements.

A magnetic particle concentrator DynaMag-2 (Cat. No: 12321D, Dynabeads®, Invitrogen™ Thermo Fisher Scientific) was employed to handle the MBs modification protocol. An MPW-65R centrifuge from MPW (Med. Instruments), a BioSan TS-100 uniform temperature incubator shaker (Thermo), a magnetic stirrer (Inbea), a Crison model Basic 20+ pH-meter and a vortex (Velp Scientifica) were also used.

Reagents and solutions

Carboxylic acid-functionalized MBs (HOOC-MBs, Dynabeads™, Cat. No.: M-270, 2.8 µm ∅, ~2×10^9 beads mL^{-1}) and commercial Blocker™ Casein in PBS (BB) were acquired from Invitrogen™ and Thermo Fisher Scientific (Cat. No. 37528), respectively. The reagents used in the MBs activation steps N-hydroxysulfosuccinimide (Sulfo-NHS), and N-(3-dimethyl-aminopropyl)-N´-ethyl-carbodiimide (EDC), were purchased from Apollo Scientific (Ref. OR307159) and Acros Organics (Ref. 171440100), respectively, while those used for the blocking steps, as well as for the amperometric measurements, ethanolamine (ETA), hydroquinone (HQ), and hydrogen peroxide (H_2O_2, 30 % v/v), were purchased from Sigma-Aldrich (Refs. 398136, H9003, and H1009, respectively).

All reagents used in the preparation of the buffer solutions were of the highest available grade and were acquired from Scharlab, including potassium chloride (Ref. PO02001000), sodium chloride (Ref. SO02271000), sodium di-hydrogen phosphate (Ref. SO03321000), di-sodium hydrogen phosphate (Ref. SO03351000), tris-hydroxymethyl-aminomethane-HCl

(Tris-HCl, Ref.TR0421000), and from GERBU Biotechnik, 2-(N-morpholino) ethanesulfonic acid (MES, Ref. 1080.1000).

The preparation of the MBs-immunoconjugates was performed with the immunoreagents provided in commercial ELISA kits. Thus, a human TIMP-1 DuoSet ELISA from R&D Systems (Cat. No. DY970) containing a recombinant human TIMP-1 standard, a mouse anti-human TIMP-1 capture antibody (CAb_{TIMP-1}) and a biotinylated goat anti-human TIMP-1 detection antibody ($b\text{-}DAb_{TIMP-1}$), and a Human GDF-15 DuoSet ELISA (Cat. No. DY957, from R&D Systems) including a recombinant human GDF-15 standard, a mouse anti-human GDF-15 capture antibody (CAb_{GDF-15}) and a biotinylated goat anti-human GDF-15 detection antibody ($b\text{-}DAb_{GDF-15}$) were employed. Streptavidin-HRP (Strep-HRP) conjugate from Roche (Cat. No. 11089153001) was used as the enzymatic tracer.

Human TNFα (Cat. No. DY210, from R&D Systems), human hemoglobin (Hb, Cat. No. H7379), IgG from human serum (hIgG, Cat. No. I2511) and albumin from human serum (HSA, Cat. No. A1653), all from Sigma-Aldrich, were evaluated as potential interferers.

The preparation of all buffer solutions (0.025 mol L^{-1} MES buffer pH 5.0; 0.1 mol L^{-1} phosphate buffer (PB) solution pH 8.0; 0.1 mol L^{-1} Tris-HCl pH 7.2; 0.01 mol L^{-1} phosphate buffer saline solution (PBS) pH 7.4, and 0.05 mol L^{-1} phosphate buffer (PB) solution pH 6.0) was performed using deionized water type I from a Millipore Milli-Q purification system (18.2 MΩ cm).

An EDC/Sulfo-NHS mixture solution (50 mg mL^{-1} each, prepared in MES buffer pH 5.0) and a 1.0 M ethanolamine (ETA) solution prepared in 100 mM PB buffer pH 8.0 were employed for MBs activation and blocking, respectively.

For the amperometric measurements, solutions of 100 mM HQ and 100 mM H_2O_2 were freshly prepared in 50 mM PB pH 6.0 right before use.

Results and discussion

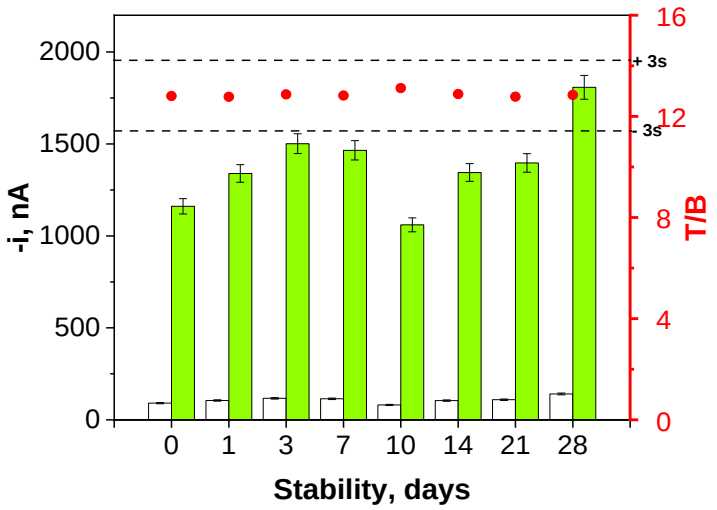

Fig. S1. Storage stability of the CAb_{TIMP-1}-MBs conjugates. Amperometric responses provided by TIMP-1 immunoplatforms prepared each control day from the CAb_{TIMP-1}-MBs (day 0) stored in filtered PBS at 4 ºC for 0 (white bars, B) and 1000 pg mL^{-1} TIMP-1 (green bars, T). T/B ratio values are displayed in red dots and control limits (dashed black lines) were set as ± 3s of the mean value of three different bioplatforms prepared on day 0.

Table S1.

t_{exp} values calculated from the comparison of the slope values of the external calibration plot and that prepared by standard additions for the determination of TIMP-1 in the assayed biological samples.

Biological sample		**Slope, nA mL pg^{-1}**	**t_{exp}***	**t_{tab} (95%, 2, 2 tails)**
Buffered standards		1.16 ± 0.03	--	
Cell extracts	SW480 (NM)	1.1 ± 0.1	0.165	
(0.25 µg)	SW620 (M)	1.2 ± 0.2	0.096	4.303
Tissue extracts	(NT)**	1.15 ± 0.08	0.149	
(0.10 µg)	(T)**	1.2 ± 0.1	0.345	

** Calculated as described in [1] by comparing the slope values. M: metastatic; NM: non-metastatic; NT: non-tumoral; T: tumoral.*
*** Samples of Patient 7 in Table S3.*

Table S2.

Determination of TIMP-1 in extracts from CRC cells (0.25 μg) with different metastatic potential using the developed immunoplatform.

Cell type	Metastatic ability	[TIMP-1], pg μg^{-1}*	M/NM	$RSD_{n=3}$, %
SW480	NM	2.14 ± 0.04	1.4	0.7
SW620	M	3.0 ± 0.2		3.0
KM12C	NM	2.37 ± 0.05	1.3	0.8
KM12L4a	M	3.01 ± 0.07		0.9
KM12SM	M	3.0 ± 0.1	1.3	1.3

**Mean value ± $t \times s/\sqrt{n}$; n = 3; α = 0.05; M: metastatic; NM: non-metastatic.*

Table S3.

Determination of TIMP-1 with the developed immunoplatform in samples of paired tissues (NT/T) extracts (0.10 μg) from patients with advanced CRC.

Patient	CRC stage	Tissue type	[TIMP-1], pg μg^{-1}*	T/NT	$RSD_{n=3}$, %
1	III	NT	11.6 ± 0.9	2.3	3.2
		T	27 ± 1		2.0
2		NT	5 ±1	6.4	9.2
		T	32 ± 3		3.3
3		NT	14 ± 4	2.4	12.6
		T	34 ± 3		3.5
4		NT	6 ±1	53.3	5.8
		T	320 ± 58		7.3
5	IV	NT	10 ± 1	4.4	5.8
		T	44 ± 2		1.9
6		NT	7 ± 1	4.9	6.6
		T	34 ± 1		0.9
7		NT	20 ± 2	1.8	3.1
		T	36 ± 4		4.3
8		NT	19 ± 2	2.47	4.4
		T	47 ± 6		5.5

**Mean value ± $ts/\sqrt{n}$; n = 3; α = 0.05. NT: non-tumoral; T: tumoral.*

Table S4.

Recovery results (mean values ± ts/√n; n = 3; α = 0.05) provided by the developed immunoplatform for protein cell extracts and CRC tissues (T/NT) spiked with 250 pg mL^{-1} of TIMP-1.

Biological sample			Found, pg mL^{-1**}	Recovery, %	$RSD_{n=3}$, %
Cell extracts		SW480	251 ± 3	100 ± 1	0.5
		KM12C	248 ± 1	99 ± 1	0.2
Tissues	Patient 3	NT*	249 ± 7	100 ± 3	1.2
		T*	251 ± 12	100 ± 5	1.9
	Patient 7	NT*	252 ± 9	101 ± 4	1.4
		T*	252 ± 9	101 ± 4	1.5

**NT: non-tumoral; T: tumoral.*

***After subtracting the endogenous content found in each sample from the overall concentration determined after sample spiking.*

Table S5.

Calculated t_{exp} values from the slope's values of the calibration plots for GDF-15 obtained with buffered solutions and in the presence of secretome samples.

Sample		Slope, nA mL pg^{-1}	t_{exp*}	t_{tab} (95%, 2, 2 tails)
Buffered standards		0.22 ± 0.01	--	4.303
Cell secretome (1/25)	SW480	0.20 ± 0.01	0.014	
	KM12C	0.21 ± 0.01	0.340	

**Estimated as described in [1] by comparing the slope values.*

Table S6.

Recovery values (mean values ± ts/√n; n = 3; α = 0.05) obtained during the analysis of plasma and cell secretome samples spiked with 250 pg mL^{-1} of the target operating with the dual analytical tool developed.

		GDF-15			TIMP-1		
Biological sample		**Concentration found, pg mL^{-1*}**	**Recovery, %**	**$RSD_{n=3}$, %**	**Concentration found, pg mL^{-1*}**	**Recovery, %**	**$RSD_{n=3}$, %**
Cell secretome	SW480	251 ± 3	100 ± 1	0.41	252 ± 12	101 ± 5	1.84
	KM12C	252 ± 4	101 ± 2	0.60	254 ± 7	102 ± 3	1.08
Plasma	2	254 ± 11	102 ± 5	1.80	255 ± 12	102 ± 5	1.95
	4	253 ± 12	101 ± 5	1.93	253 ± 6	101 ± 2	0.89
	6	253 ± 11	101 ± 4	1.68	255 ± 10	102 ± 4	1.62

**After subtraction of the endogenous content found in each sample from the overall concentration determined after sample spiking.*

Reference

[1] J. Andrade, M. Estévez-Pérez, Statistical comparison of the slopes of two regression lines: a tutorial, Anal. Chim. Acta 838 (2014) 1–12, https://doi.org/10.1016/j.aca.2014.04.057.