



Determining and characterizing circulating nucleosomes in advanced cancer with electrochemical biosensors assisted by magnetic supports and proteomic technologies

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ABSTRACT

Measuring and monitoring plasma nucleosomes (small fragments of chromosomes released into the blood during cell death), and their proteomic profiles is a promising approach for improved early detection, diagnosis, and prognosis of cancer diseases as well as for disease and therapy follow-up, contributing to more personalized and effective cancer care. Early detection is imperative in colorectal cancer (CRC), as it has been proven to significantly improve patient outcomes. Indeed, in CRC, blood circulating nucleosome levels and their histone alterations have been correlated with tumor stage and the presence of metastasis. Moreover, they constitute promising markers for CRC monitoring and prognosis in a minimally invasive way. The current technologies used for their detection suffer from notable drawbacks such as non-selective identification and quantification of unknown cancer-relevant proteomic trademarks, expensive procedures, and variable results related to staff experience. Taking advantage of the sensitive, fast, cost-effective, and reliable methodologies that electroanalytical technologies offer for the determination of multilevel biomarkers in liquid biopsies, we report in this paper the first electrochemical immunoplatfrom for the isolation and determination of circulating nucleosomes in plasma using an anti-H3.1 histone variant, integrated with proteomics insights to confirm nucleosomes isolation and identify associated proteins with potential as CRC biomarkers. The developed bioplatfrom was used to analyze 0.5 µg of nuclear extracts from CRC cells with different metastatic potential as well as 1/5 diluted plasma samples, demonstrating the suitability to effectively discriminate CRC patients in advanced stages from healthy individuals through liquid biopsy.

1. Introduction

Nucleosomes, complexes of DNA, histone proteins, and associated proteins, are released into the bloodstream during cell death processes such as apoptosis and necrosis due to digestion of the DNA linking adjacent nucleosomes (approximately 20–80 base pairs (bp) in length) bound by histone H1 (Holdenrieder et al., 2008; Silk et al., 2017; Ras-mussen et al., 2018; Tsoneva et al., 2023). They consist of a histone octamer core (composed of two H2A, H2B, H3, and H4 histones) with small double-stranded DNA chains of approximately 147 bp wrapped

around it. When these disk-shaped zwitterionic nanoparticles (diameter 11 nm, height 5.5 nm) with a molecular weight of approximately 206 kDa, and equally contributed by DNA and histone components, are released from cancer cells, they may host diverse histone post-translational modifications (PTMs) which alter the conformation and the interaction properties of the nucleosome (Grolleau et al., 2023; Tsoneva et al., 2023; Wang et al., 2024). Elevated levels of circulating nucleosomes have been detected in patients with various types of cancer, including non-Hodgkin lymphoma (NHL) and colorectal cancer (CRC) (Holdenrieder et al., 2008; Holdenrieder et al., 2014; Rahier et al.,

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2017; Van den Ackerveken et al., 2021; Van den Ackerveken et al., 2023; Wang et al., 2024; Zhou et al., 2024). These structures not only reflect tumor burden but also carry specific epigenetic modifications that provide valuable insights for cancer diagnosis and monitoring (Van Den Ackerveken et al., 2023; Wang et al., 2024).

A recent study investigated the epigenetic profiles of circulating nucleosomes containing the H3.1 histone variant in NHL patients (Van Den Ackerveken et al., 2023). The results showed significantly higher levels of these nucleosomes compared to healthy donors. Additionally, these authors identified eight PTMs that distinguished patients from healthy controls. Therefore, both the level of circulating nucleosomes and their PTMs can serve as potential biomarkers for NHL diagnosis and disease monitoring (Van Den Ackerveken et al., 2023).

CRC ranks as the third most common cancer globally, with approximately 1.92 million new cases and over 900,000 related deaths in 2022 (World Cancer Research Fund, 2025). Early detection significantly enhances patient outcomes, underscoring the need for effective screening methods (Kuipers et al., 2015; Simon, 2016; Hong, 2018; Ma et al., 2024). In CRC, circulating nucleosome levels and histone modification alterations correlate with tumor stage and metastasis presence (Gezer and Holdenrieder, 2014; Holdenrieder et al., 2014; Rahier et al., 2017; Rasmussen et al., 2018). While not tumor-specific, their dynamics provide valuable information for monitoring therapeutic efficacy and may help guide early treatment adjustments (Holdenrieder et al., 2014; Rahier et al., 2017; Wang et al., 2024).

In this context, the detection and analysis of circulating nucleosomes, their epigenetic modifications, and associated proteins represent a promising approach for identifying non-invasive cancer biomarkers and to enhance early detection, prognosis, and disease monitoring, contributing to more personalized and effective cancer care (McAnena et al., 2017).

Proteomic analyses have delved into the epigenetic profiles of circulating cell-free (cf) nucleosomes in CRC patients. A study measuring 12 epigenetic cf nucleosome epitopes in serum identified a panel of four plasma nucleosomes able to discriminate with high sensitivity CRC patients from healthy controls, even at early disease stages (Rahier et al., 2017). These results suggest that specific circulating nucleosome structures and/or associated proteins specific to CRC can serve as promising biomarkers for the detection of the disease by liquid biopsy.

Prompt detection, diagnosis, and treatment are key factors to diminish CRC mortality and morbidity. Colonoscopy, the gold standard for CRC detection, is an invasive procedure scarcely accepted by patients and restricted by economic and technical issues. The fecal occult blood test (FOBT) and the fecal immunochemical test (FIT) have entered clinical use, while researchers work on the development of minimally invasive screening based on blood tests (Wang et al., 2024). Due to their early diagnostic significance, the determination of circulating nucleosomes and their histone modifications is gaining interest in cancer screening. Methods for detecting nucleosomes include enzymatic digestion, chemical cleavage, immunoprecipitation, Enzyme-linked Immunosorbent Assays (ELISAs), DNA sequencing, and computational tools (Fang et al., 2022). ELISA kits are mainly used for detecting nucleosomes in cell cultures and body fluids. The assays are based on two monoclonal mouse antibodies directed against epitopes on DNA and histone components that specifically capture mono- and oligonucleosomes originated in the eukaryotic cell nucleus (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics) (Holdenrieder et al., 2001a, 2001b; Fahmueller et al., 2012). These immunoassays are suitable for the quantification of circulating histones/nucleosomes or specific modifications on histones in fluid samples but less selective for the identification and quantification of unknown cancer-relevant associated proteins and histone PTMs (Tsoneva et al., 2023). Other technologies used in the detection of circulating histones include proteomics involving immunoprecipitation followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) for multiple reaction monitoring, which is expensive and requires experienced analysts. Also,

ImageStream, a multi-channel imaging technology that combines phenotypic sensitivity, multiplex flow cytometry, and the visual power of microscopy has been used to detect multiple biomarkers, including histones and cancer cells, using low amounts of sample with a relatively low cost although showing inter-observer variability, and reproducibility directly related with the level of experience of the analyst (Tsoneva et al., 2023). PCR-based methods requiring more patient material and a single-molecule multiparametric assay to comprehensively analyze the epigenetics of plasma-isolated nucleosomes (EPINUC), DNA methylation, and cancer specific protein biomarkers have also been described (Fedyuk et al., 2022) (Wang et al., 2024).

Advances in detection methodologies have led to the development of electrochemical biosensors for multilevel biomarkers in liquid biopsy (Campuzano and Pingarrón, 2023). Electrochemical biosensors allow fast, sensitive, and cost-effective analyses, and have been demonstrated suitability for challenging clinical applications (Campuzano and Pingarrón, 2023). However, to date, this type of technology has not been explored for the isolation and determination of circulating nucleosomes. Additionally, integrating proteomic insights with biosensor technology holds significant promise for enhancing cancer screening and monitoring (Williams and Addona, 2000).

In this study, we report a multilevel approach combining the insights of labeled electrochemical biosensors to selectively capture and determine circulating nucleosomes using an anti-H3.1 histone variant involving a sandwich immunoassay with enzymatic labeling, generating amperometric signals at screen-printed carbon electrodes (SPCEs), with those of label-free quantitative proteomics to confirm the isolation of nucleosomes and identify nucleosome and nucleosome-associated proteins. The evaluation of this innovative approach in real-life CRC scenarios highlights the potential of both the identified nucleosomes and nucleosome-associated proteins and the synergistic combination of the two types of cutting-edge technologies to contribute to the prognosis and minimally invasive monitoring of this neoplasia with high mortality and prevalence.

2. Materials and methods

Apparatus and electrodes, Reagents and solutions, Bioassay implementation on magnetic beads (MBs), Amperometric measurements, Cells and Human samples, Characterization of captured nucleosomes by proteomic technologies (Nucleosome proteome digestion, LC-MS/MS analysis, MS data analysis, Bioinformatic, ROC curves, and statistical analysis) are described in detail in the Supporting Information.

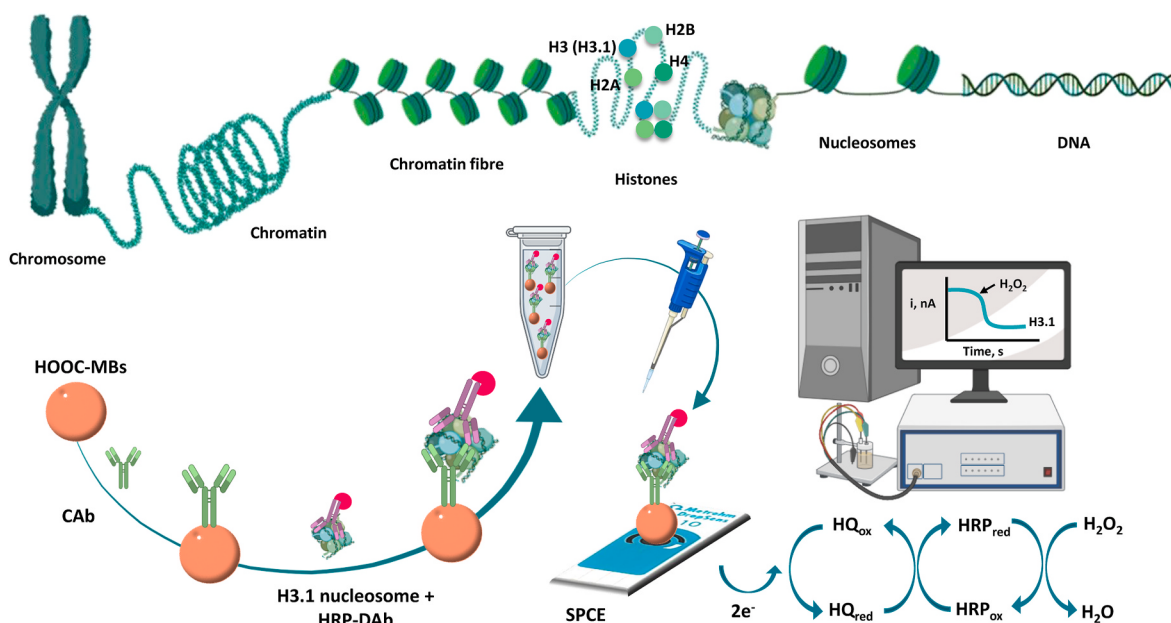
3. Results and discussion

Scheme 1 shows the rationale of the developed bioplatfrom for the determination of nucleosomes using H3.1 nucleosomes as model. Briefly, the method involved the use of two antibodies, a capture antibody that recognizes a specific peptide of histone H3.1 and a detector antibody conjugated with HRP that recognizes intact nucleosomes, that is, both the protein core and the wrapping DNA.

As seen in **Scheme 1**, quantification of nucleosomes was accomplished by trapping on the working electrode (WE) of a SPCE the nucleosome complexes captured on MBs. Amperometric measurements involved the variation in the cathodic current recorded in stirred solutions using the HRP/H₂O₂/hydroquinone (HQ) system by applying a potential of −0.20 V (vs Ag pseudo-reference electrode).

3.1. Optimization of experimental variables

Key experimental variables affecting the preparation of the immunoconjugates on MBs and, therefore, the determination of nucleosomes, were optimized using the conditions previously established for amperometric transduction using the HRP/H₂O₂/HQ system (applied potential, pH, and composition of the supporting electrolyte and H₂O₂ and HQ



Scheme 1. Fundamentals of the immunoplateform assisted by MBs for the determination of nucleosomes (H3.1 nucleosomes as model) using amperometric transduction at SPCEs.

concentrations (Eguílaz et al., 2020)). Larger target-to-blank signal ratios (T/B) obtained either in the presence of a fixed standard concentration (2500 ng mL⁻¹, T) or in its absence (B) were used as the selection criterion.

The variables evaluated included the concentration and incubation time with the H3.1 CAb solution for the preparation of the immunocaptors (H3.1 CAb-MBs), the number of steps involved in the preparation of the bioconjugates, and the incubation time with the mixture of the standard and the detection antibody for the capture and enzymatic labeling of the nucleosomes on the CAb-MBs. The results obtained are shown in Fig. S2 and summarized in Table S1 (both in the Supporting Information).

According to the results displayed in Fig. S2a, the T/B ratio increased with the concentration of H3.1 CAb. Considering both the high sensitivity required and the cost per analysis, we decided to implement the immunoplateform using a H3.1 CAb concentration of 100 µg mL⁻¹. However, if higher sensitivity was needed, modification of activated MBs with a larger H3.1 CAb concentration would be possible.

As can be seen in Fig. S2b, an incubation time of 30 min of the activated particles in a 100 µg mL⁻¹ H3.1 CAb solution provided a better T/B ratio for their covalent immobilization. This ratio slightly decreased for longer incubation times, which can be attributed to a worse efficiency of the target antigen recognition due to steric hindrances for times over 30 min leading to immobilization of a lot of CAb molecules on MBs (Eletxigerra et al., 2014). The results obtained in the absence of H3.1 CAb ("bars 0" in Fig. S2a and b) allow concluding that no significant nonspecific adsorptions of nucleosomes on unmodified MBs were apparent, confirming their capture on the H3.1 CAb through the histone core.

Regarding the number of steps involved in the preparation of the bioconjugates (Fig. S2c), two different protocols were compared. Both protocols used 30 min incubation steps and started from prepared H3.1 CAb-MBs. Protocol I implied a single incubation step of the H3.1 CAb-MBs with a mixture solution containing the nucleosome standard and the HRP-DAB (Fig. S2c, bars I); therefore, the capture and enzymatic labeling of the nucleosomes was performed in a single step. However, protocol II separated these two processes into two successive incubation steps, first in the nucleosome standard solution, and then in the HRP-DAB solution (Fig. S2c, bars II). A better T/B ratio was observed using

the one-step protocol, which, obviously, is also convenient in terms of simplicity and assay time.

The results for the incubation time of the H3.1 CAb-MBs with the mixed solution of the nucleosome standard and HRP-DAB (Fig. S2d) confirmed that a 30 min incubation was enough to obtain a better T/B ratio, so it was selected for the following work. The decrease in the responses observed with long incubation times can be attributed to aggregation and/or agglutination phenomena of the bioreagents in solution, affecting the efficiency of the capture and/or enzymatic labeling processes.

3.2. Analytical and operational characteristics and selectivity

The calibration plot constructed for the amperometric determination of nucleosome standards between 0 and 10000 ng mL⁻¹ and under the optimized experimental conditions is depicted in Fig. 1. A linear

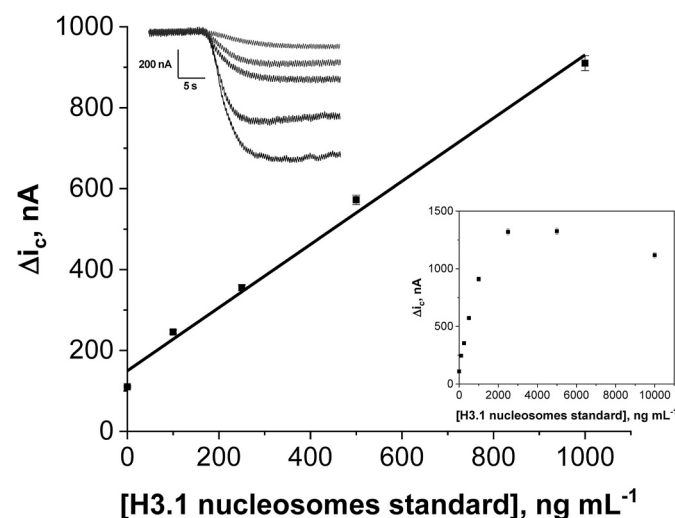


Fig. 1. Calibration plot, linear range, and recorded amperometric traces obtained with the developed immunoplateform for 0, 100, 250, 500, and 1000 ng mL⁻¹ H3.1 nucleosomes standard.

dependence between the measured current and the target standard concentration was found between 26 and 1000 ng mL⁻¹ ($R^2 = 0.9912$), which fitted to the equation $\Delta i_c, \text{nA} = (0.78 \pm 0.04) \text{nA mL ng}^{-1} [\text{H3.1 nucleosomes standard}] + (150 \pm 22) \text{nA}$. A limit of detection (LOD) of 7.82 ng mL⁻¹ was calculated according to the $3 \times s_b/\text{slope}$ criterion, where s_b is the standard deviation of 10 B signals.

A relative standard deviation (RSD) of 2.0 % was calculated from the amperometric measurements provided by different bioplatforms prepared on the same day ($n = 10$, 500 ng mL⁻¹ of the H3.1 nucleosomes standard), which confirmed the good reproducibility of the measurements provided by the assay. It is also important to mention that CAB-MBs bioconjugates were stable for 15 days when stored resuspended in filtered PBS at 4 °C until use (Fig. S3 in the Supporting Information).

The potential interfering effect of the presence of human serum IgG (hIgG), human hemoglobin (Hb) and human serum albumin (HSA) proteins was checked by comparing the amperometric responses measured for 0 and 500 ng mL⁻¹ H3.1 nucleosome standard in the presence of these proteins at their usual concentrations found in the serum of healthy individuals (specified in caption of Fig. S4 in the Supporting Information).

As already reported for other sandwich immunoassays implemented on MBs (Muñoz-San Martín et al., 2022; Tejerina-Miranda et al., 2024), both hIgG and Hb interfered at the highest concentrations tested (1 and 5 mg mL⁻¹, respectively). The interference of hIgGs can be attributed to the prevalence in serum of human anti-mouse antibodies (HAMAs) which are able to react with the heavy chain of mouse immunoglobulins (Koshida et al., 2010), as the used H3.1 CAB. HAMAs are heterophil antibodies existing in human serum that can interfere with immunoassays by bridging or blocking the involved antibodies leading to misdiagnosis and unnecessary treatments. Although the administration of murine monoclonal antibodies induces HAMA response, according to different studies, HAMAs are expressed in 11.7 % of serum samples tested in routine examinations (Koshida et al., 2010), and are prevalent in the serum of CRC patients, even before monoclonal antibody administration (Goto et al., 2010). The interference of Hb is due to its peroxidase activity (Grigorieva et al., 2013), which is relevant because of the redox system (H₂O₂/HQ) used in the amperometric transduction. However, both interferences became negligible when hIgG and Hb were tested at 10-fold diluted concentrations. As will be shown in the next section, these compounds do not represent any problem to determine H3.1 nucleosomes in plasma samples with the developed bioplatfrom.

As commented in the Introduction section, circulating nucleosomes in cell cultures and body fluids, such as serum and plasma, are currently detected mainly using ELISAs, prototypes involving MBs and chemiluminescence immunoassays (ChLIAs), PCR methods, or the EPINUC assays (Wang et al., 2024). ELISAs (e.g. Cell Death Detection ELISA^{PLUS} from Roche and NuQ-X ELISA Assay from Volition) and MBs-ChLIAs (Nu.Q® prototype immunoassays) detect epitopes on DNA and histone components (Al-Shuneigat et al., 2011; Fahmueller et al., 2012; Holdenrieder et al., 2014; Bauden et al., 2015; Rahier et al., 2017; Rasmussen et al., 2018; Grolleau et al., 2023; Berman et al., 2024), and PCR-based methods target the cfDNA existing in the form of mono- or oligo-nucleosomes (Holdenrieder et al., 2005) requiring more patient material than ELISAs. On the other hand, the EPINUC is a single-molecule multiparametric assay to comprehensively analyze the epigenetics of plasma-isolated nucleosomes, which allowed for high-resolution detection of six active and repressive histone modifications and their ratios and combinatorial patterns on millions of individual nucleosomes by single-molecule imaging (Fedyuk et al., 2022). It demonstrated cancer detection with high accuracy and sensitivity, even at early stages, by analyzing a cohort of plasma samples from CRC, and pancreatic cancer patients, and healthy individuals. Furthermore, its combination with direct single-molecule DNA sequencing revealed the tissue of origin of colorectal, pancreatic, lung and breast tumors, providing multilayered information of potential clinical relevance from limited (<1 mL) liquid biopsy material.

It is important to note that, according to its specifications, the Nu.Q® Discover H3.1 ELISA Assay-RUO (Ref. 1001-01-03, from Volition) has a reportable range of 22.7–650 ng mL⁻¹ and a LOD of 9.1 ng mL⁻¹ H3.1-nucleosomes, which is slightly larger than that achieved with the developed bioplatfrom (7.82 ng mL⁻¹). ELISA implies a longer assay (290 vs. 30 min from preparation of H3.1 CAB-ELISA plate and H3.1 CAB-MBs, respectively) and requires more incubation steps (3 vs. 1, respectively, excluding the washing steps) to perform the determination. In addition, ELISAs use microplate readers that are less affordable and portable than amperometric transducers.

It is worth noting that ELISA kits for the determination of circulating nucleosomes in connection with CRC, were exploited, among others, by Al-Shuneigat et al. and Fahmueller et al. proving the significant increase in the levels of serum nucleosomes in CRC patients (Al-Shuneigat et al., 2011) compared to pretherapeutic and healthy mean values (191 and 36 ng mL⁻¹) (Fahmueller et al., 2012). Both authors used the cell death detection ELISA^{PLUS} from Roche, studying the association of high nucleosome levels with advanced stages of colorectal carcinoma (Al-Shuneigat et al., 2011). Other interesting published contributions described a significant reduction in the level of circulating nucleosome-associated 5-methylcytosine in CRC relative to healthy individuals (Holdenrieder et al., 2014), a signature of four nucleosome epitopes (H2AK119Ub, H3K9Ac, H4K20me3, and the global level) to detect CRC at early stages (stages I and II) with high sensitivity (Rahier et al., 2017), and the serum levels of circulating cf nucleosomes among patients with CRC as stage-independent and therefore potentially promising in early detection of CRC (Rasmussen et al., 2018).

The work reported by Van den Ackerveken et al. relying on the capture of intact circulating nucleosomes using an immunoprecipitation method followed by LC-MS/MS (Nu.Q Capture-MS) for the detection and the quantification of circulating nucleosomes and their associated histone epigenetic modifications in plasma of CRC patients (Van den Ackerveken et al., 2021) and NHL (Van den Ackerveken et al., 2023) should also be mentioned. In both cases, authors found significantly higher concentrations of circulating H3.1 nucleosomes in plasma samples of NHL and CRC patients compared to samples from healthy individuals (Van den Ackerveken et al., 2021; Van den Ackerveken et al., 2023). Within this context, the developed bioplatfrom points out as an interesting alternative to determine H3.1 nucleosomes because of its ease of operation, outstanding sensitivity, low reagents expenditure and cost, and possibility of implementation in the point-of-care (POC) due to the type of substrates, instrumentation and detection technique used for transduction.

3.3. Application to the analysis of real samples

With the aim of validating the results provided by the bioplatfrom, the controls supplied in the commercial ELISA kit were analyzed. To do this, the measurements obtained with the bioplatfrom for the undiluted controls supplemented with the HRP-DAB were interpolated into a calibration graph constructed with the standards provided in the kit. The results obtained ($\alpha = 0.05$, $n = 3$, Table S2) confirmed an acceptable accuracy of the results provided by the developed biotool. The slight discrepancies observed for control 1 can be attributed to the use of different CABS and analytical protocols employed by the bioplatfrom and the ELISA kit.

The developed bioplatfrom was used to analyze 0.5 µg of nuclear extracts from CRC cells with different metastatic ability. Fig. 3 shows as all the samples tested exhibited amperometric responses larger than that of the blank, confirming the presence of H3.1 nucleosomes in all the extracts. According to the responses obtained, the concentration of H3.1 nucleosomes was higher in the nuclear extracts of metastatic (SW620, KM12SM, and KM12L4a) compared to non-metastatic (SW480 and KM12C) cells. These results agree with that previously reported showing increased total levels of nucleosomes in the blood associated with tumor burden and malignancy progression in CRC (Al-Shuneigat et al., 2011;

Rahier et al., 2017).

Since in CRC circulating nucleosome levels and PTMs are described as being associated with tumor stage and metastasis, the developed immunoplatfrom was faced with the challenging determination of H3.1 nucleosomes in 28 plasma samples, 10 from healthy individuals and 18 from CRC patients in advanced stages (10 from stage III and 8 from stage IV). The determination of H3.1 nucleosome was carried out by performing a simple 1/5 plasma dilution, to allow signals to be within the linear range of the biosensor and minimize the possible matrix effect, and interpolating the amperometric responses obtained for the diluted samples into the calibration plot constructed with standards (Fig. 2). The results obtained are shown in Fig. 3.

According to the results obtained, the concentration of H3.1 nucleosomes was significantly higher in patients with advanced CRC (CRC III: mean 189.9 ng mL^{-1} , range $3.9\text{--}658.3 \text{ ng mL}^{-1}$; CRC IV: mean 144.8 ng mL^{-1} , range $35.5\text{--}264.1 \text{ ng mL}^{-1}$) than in healthy individuals (mean: 82.8 ng mL^{-1} , range: $0.0\text{--}122.5 \text{ ng mL}^{-1}$). It must be noted that the obtained concentrations agree with those reported by Herzog's group, using the Nu. Q® Discover H3.1 ELISA Assay-RUO Kit (Ref. 1001-01-03, Volition), in the analysis of plasma samples from healthy individuals (mean: $56.18 \pm 29.28 \text{ ng mL}^{-1}$) and non-Hodgkin lymphoma patients ($582.3 \pm 564.4 \text{ ng mL}^{-1}$) (Van den Ackerveken et al., 2023).

These results show the potential of the developed immunoplatfrom against conventional techniques, such as ELISA and blotting, to quantify plasma levels of cf H3.1 nucleosomes. The determination can be made in just 30 min starting from the preparation of H3.1 CAB-MBs. Additionally, we calculated ROC curve analyses to check the ability of the bioplatfrom to discriminate between healthy individuals and CRC patients in advanced stages (Fig. 4). As can be seen, significant discrimination was achieved with area under curve (AUC) values and sensitivity and specificity up to 74 %, 70 %, and 100 %, respectively.

Moreover, to assess the accuracy of the results provided by the developed bioplatfrom, recovery studies were carried out by supplementing two representative plasma samples from a healthy individual and a patient with CRC III with a known amount of standard H3.1 nucleosomes (250 ng mL^{-1}). The recovery values obtained, applying the same protocol as for the analysis of not supplemented samples, were 107.1 and 107.3 %, respectively, thus confirming the accuracy of the obtained results.

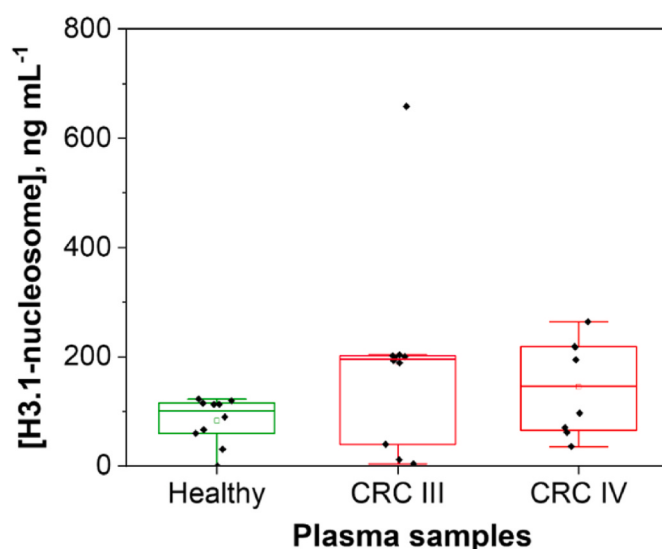


Fig. 3. Determination of H3.1 nucleosomes with the developed bioplatfrom for plasma samples grouped into pools of healthy individuals and CRC patients in stages III and IV.

To confirm the isolation of circulating nucleosomes and further investigate the role of nucleosomes and nucleosome-associated proteins as plasma biomarkers for CRC, plasma H3.1 nucleosomes captured using H3.1 CAB-MBs were characterized using cutting-edge proteomics technologies.

Following nucleosome trypsin digestion, protein identification and quantification were performed by label-free LC-MS/MS proteomics using an Orbitrap Astral Mass Spectrometer. Data normalization was successfully achieved, ensuring consistent protein input across all biological replicates (Fig. 5a). Overall, 1457 proteins were commonly identified in at least 60 % of samples for each condition. Remarkably, H3.1 histone was consistently identified for all the 14 analyzed samples (5 from healthy individuals, 5 from CRC stage III, and 4 from CRC stage IV patients), along with other associated histones, ribonucleoproteins and chaperons (Fig. 5b). These results confirmed the successful capture of plasma nucleosomes and validated the results obtained using the developed bioplatfrom. A similar behavior among biological replicates, except for one CRC patient at stage III sample, was observed with suitable discrimination between samples of healthy individuals and CRC patients that additionally confirmed the results obtained with the bioplatfroms with discrimination between groups according to the nucleosomes content (Fig. 5c). The identified proteins underwent functional, biological pathway and cellular component enrichment analyses using STRING (2025 database; Szklarczyk et al., 2025) (Fig. 5d). The obtained results highlighted protein involvement in nucleic acid, nucleoside phosphate and nucleotide binding, supporting the nucleolar role of plasma nucleosomes. Additionally, several of the most enriched biological pathways were related to translation, further corroborating the nuclear relevance of these plasma-captured molecules. Furthermore, cellular component analysis revealed extracellular protein identifications, demonstrating a nuclear-extracellular association of secreted nucleosomes.

In addition, proteomic analysis identified 18 unique proteins in plasma nucleosomes isolated from stage III and IV CRC patients: ATP13A1, BUD31, FAM83D, GALNT1, GPN1, GTF2F1, HERC4, KCNJ13, MRPL38, NAA50, NBAS, PAXBP1, RPA3, SDC2, TMEM109, TRAPPC9, UBE3C, and XPO5. According to the information available in the databases, BUD31, FAM83D, GPN1, and NAA50 are noteworthy for future research as potential minimally invasive diagnostic/prognostic factors of CRC. In fact, the databases state that these four proteins are prognostic markers in different types of cancer: liver hepatocellular

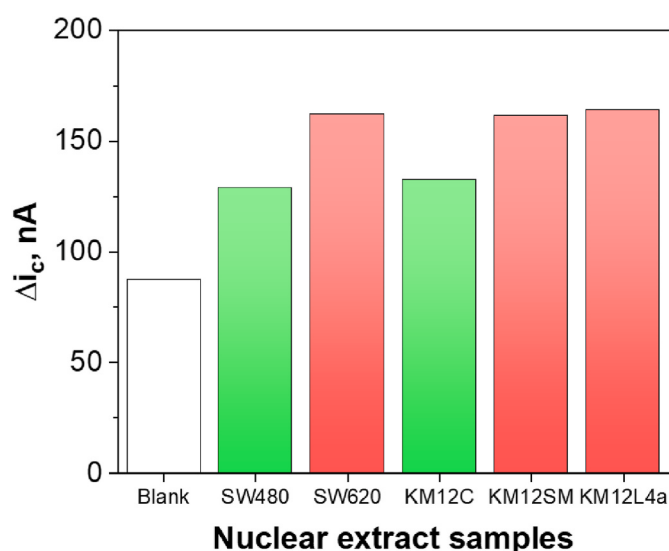


Fig. 2. Amperometric responses provided by the bioplatfrom in the absence (Blank) and in the presence of $0.5 \mu\text{g}$ of nuclear extract from CRC cells with different metastatic properties (green bars, non-metastatic cells; red bars, metastatic cells). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

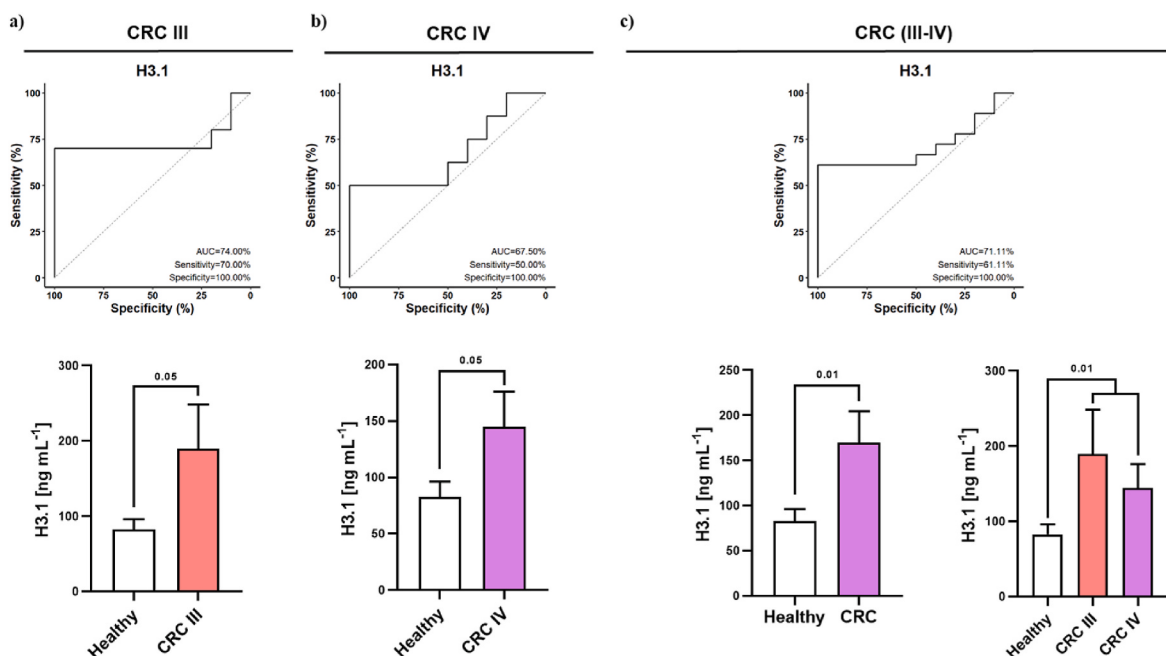


Fig. 4. ROC curve analyses of the H3.1 nucleosome concentrations of the 28 CRC plasma samples obtained from the developed immunoplatfrom. Results were grouped according to the patients' CRC stage (III or IV) and compared against healthy individuals. Both CRC III **a)** and CRC IV **b)** stages were associated with a significant increase of H3.1 nucleosomes. **c)** AUC, sensitivity, and specificity were calculated with R. Results are plotted as mean values $\pm$ standard error of the mean (SEM). Student's *t*-test values were calculated and considered significant when $p \leq 0.05$.

carcinoma (NAA50, GPN1, FAM83D, and BUD31), lung adenocarcinoma (NAA50, GPN1, and FAM83D), renal cancer (BUD31, FAM83D, and GPN1), and pancreatic adenocarcinoma (NAA50 and FAM83D). However, neither the relationship of NAA50, GPN1, and BUD31 proteins with CRC nor the presence of NAA50, BUD31, and FAM83D in plasma have been described.

All these results, constituting the first comprehensive investigation of circulating cell-free H3.1 nucleosomes and their associated proteins carried out using the tandem of cutting-edge electroanalytical and proteomic technologies, open the door to improve nucleosomes specificity in cancer detection by discovery, determination, and validation of specific signatures comprising both nucleosomes and proteomic markers characteristic of specific oncological scenarios.

4. Conclusions

This study reports the first multilevel approach integrating labeled electroanalytical technologies with label-free quantitative proteomics to selectively capture, quantify, and characterize circulating nucleosomes, as well as identifying characteristic nucleosome-associated proteins. By targeting the H3.1 histone variant, the developed method enables the determination and proteomic characterization of plasma nucleosomes, highlighting the potential of these nucleosomes and nucleosome-associated proteins as promising markers for CRC monitoring and prognosis in a minimally invasive way.

The developed electroanalytical immunotechnology enabled the simple and rapid detection of H3.1 nucleosomes (LOD value of 7.82 ng mL⁻¹) and was successfully applied to the analysis of challenging samples, effectively discriminating against the metastatic potential of CRC cell lines and assessing H3.1 nucleosome expression levels in plasma samples of healthy individuals and patients with advanced CRC requiring small amounts of samples (0.5 μ g cell extracts) and minimum sample treatment (1/5 diluted plasma samples). All the obtained results highlight the developed methodology as a sensitive, easy-to-operate, and rapid alternative for the accurate determination of H3.1 nucleosomes.

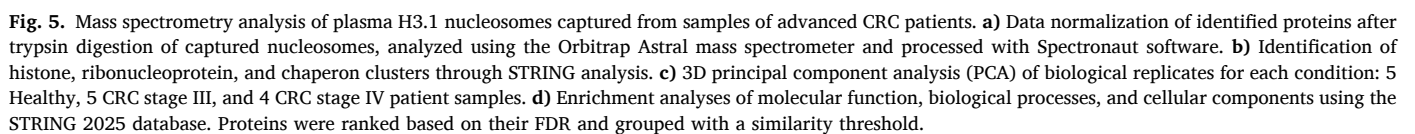
Furthermore, the analysis of isolated plasma nucleosomes using

advanced proteomic techniques proved their specific isolation, and clear differences in their circulating levels between healthy individuals and advanced CRC patients. It also allowed the identification of ten extracellular proteins, four of them (NAA50, GPN1, FAM83D, and BUD31) described in databases as prognostic factors in other cancers different from CRC. All these results confirmed the usefulness of nucleosomes and particular extracellular proteins as prognostic CRC biomarkers in liquid biopsy.

Importantly, the two technologies used in this multi-level approach feed back into each other, offering a multitude of future possibilities. Among them, the exploration of other types of patient cohorts (with early CRC or premalignant lesions) and the performance of longitudinal studies, for example, following metastatic CRC patients before and during treatment, and multicenter validation studies can be mentioned. Furthermore, the versatility of electroanalytical technologies for both the determination of proteins and their post-translational modifications, and epigenetic markers make them particularly well suited to detect and determine the clinical potential of these identified nucleosomal markers, and to implement multiplexed and multiomic technologies for their simultaneous determination in liquid biopsy samples. Besides improving tumor specificity in cancer detection, their unique capabilities will maximize the information obtained from limited reagents and sample volumes, thus improving efficiency and accelerating the accurate and early diagnosis, prognosis, and therapeutic monitoring of complex and heterogeneous diseases such as cancer and even contributing to the design of personalized treatments.

CRedit authorship contribution statement

Sandra Tejerina-Miranda: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Elisa Carral-Ibarra:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Maria Gamella:** Writing – review & editing, Writing – original draft, Supervision, Investigation. **Ana Montero-Calle:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Maria Pedrero:** Writing – review & editing, Writing – original draft, Supervision. **José M. Pingarrón:** Writing – review & editing, Writing – original



Novelty statement BB

This study reports the first multilevel approach integrating electrochemical biosensors with quantitative proteomics to selectively capture, quantify, and characterize circulating nucleosomes. By targeting the H3.1 histone variant, the developed method enables the detection and

analysis of nucleosomes and associated proteins providing a powerful tool for differentiating CRC from non-cancerous conditions. The developed bioplatfrom enabled the detection of H3.1-nucleosomes with a LOD value of 7.82 ng mL^{-1} in just 30 min counting since the preparation of H3.1 CAB-MBs, which is an assay time notably shorter (30 min vs. 230 min) and less complex (1 vs. 3 assay steps) compared to the ELISA kit that uses the same bioreagents. The bioplatfrom was successfully applied to the analysis of challenging samples, effectively discriminating against the metastatic potential of CRC cell lines and assessing H3.1 nucleosome expression levels in plasma samples of healthy individuals and patients with advanced CRC. The method needs small amounts of sample (0.5 μg of nuclear extracts from CRC cells) and requires minimum sample treatment (1/5 diluted plasma samples). The accuracy of the measurements was successfully assessed by the analysis of control nucleosome standards and spiked plasma samples. All the obtained results highlight the potential of the developed methodology as a sensitive, easy-to-operate, and rapid alternative for the determination of H3.1 nucleosomes, with potential application in POC devices. Furthermore, the analysis of plasma nucleosomes from CRC patients isolated using the bioplatfrom was carried out using advanced proteomic techniques. Nucleosome proteins were identified in the samples, supporting the results presented here, and confirming that nucleosomes are mostly released from cancer cells and circulate in the blood, and they have been effectively isolated with the developed bioplatfrom. Clear differences in nucleosome levels between healthy individuals and advanced CRC patients were observed. Moreover, ten extracellular proteins were also identified, with four of them (NAA50, GPN1, FAM83D, and BUD31) described in databases as prognostic factors in other cancers different from CRC. All these results confirmed the usefulness of nucleosomes and particular extracellular proteins as prognostic CRC biomarkers in liquid biopsy. This novel integration of electrochemical biosensing with proteomics represents a significant breakthrough in cancer diagnostics, paving the way for a better understanding of disease progression. Considering the successful detection and analysis of plasma nucleosomes in CRC patients, the developed bioplatfrom paves the way for broader applications. Since apoptotic cells release nucleosomes, this technology may also be applied to measure apoptotic cell death in cellular systems, and to increase scientific knowledge about the role of inappropriate regulation of apoptosis in many diseases, such as cancer, AIDS, autoimmunity, Alzheimer's disease, etc. The ability to detect these nucleosomal markers with high sensitivity can provide deeper insights into disease evolution. Further refining of the developed bioplatfrom may also expand its clinical utility, while exploring additional biomarkers which may enhance diagnostic accuracy, reduce costs, and enable earlier detection towards a more precise monitoring, and better patient outcomes.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2025.117582>.

Data availability

Data will be made available on request.

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