Among the methods to enhance the sensitivity of nuclear magnetic resonance (NMR) spectroscopy, small-diameter NMR coils (microcoils) are promising tools to tackle the study of mass-limited samples. Alternatively, hyperpolarization schemes based on dynamic nuclear polarization techniques provide strong signal enhancements of the NMR target samples. Here we present a method to effortlessly perform photo-chemically induced dynamic nuclear polarization in microcoil setups to boost NMR signal detection down to sub-picomole detection limits in a 9.4T system (400 MHz $^1$H Larmor frequency). This setup is unaffected by current major drawbacks such as the use of high-power light sources to attempt uniform irradiation of the sample, and accumulation of degraded photosensitizer in the detection region. The latter is overcome with flow conditions, which in turn open avenues for complex applications requiring rapid and efficient mixing that are not easily achievable on an NMR tube without resorting to complex hardware.
Despite its low sensitivity, nuclear magnetic resonance (NMR) spectroscopy is undoubtedly one of the most powerful analytical techniques to obtain unique information on (macro)molecular composition, structure and dynamics. Strategies to improve sensitivity comprise use of extreme magnetic fields, cryoprobe technologies and dynamic nuclear polarization (DNP) techniques, which however all go hand in hand with enormous costs and requirements for high tech equipment. Alternatively, microcoils have proven to be a most cost-effective approach to tackle mass sensitivity on the basis that sensitive signal detection depends on the sample/coil volume ratio; however, concentration sensitivity still remains an issue. Here we show that the combination of microcoils with photo-chemically induced dynamic nuclear polarization (photo-CIDNP) provides a breakthrough in both mass and concentration sensitivity enhancements of small-volume NMR and a concomitant renaissance of the photo-CIDNP technique, that to date has been limited to specialized labs. This setup provides low-cost components and excellent performance at the moderate magnetic field strengths of 9.4T, rivaling performance limits of state-of-the-art cryoprobes at much higher field strengths.

Miniaturized NMR coils are promising tools to both enhance the amplitude of the NMR signal and to enable the study of mass-limited samples in a number of applications. In particular, glass chips with integrated planar spiral microcoils have proven most versatile, and have enabled us the study of supramolecular interactions and online monitoring of isothermal and non-isothermal chemical reactions. Still, glass-based microfluidic NMR chips require clean-room technology and hence are relatively time-consuming and expensive in manufacturing, hampering the advancement and more wide-spread use of small-volume NMR technologies. In this regard, 3D-printed and polymer-based microfluidic NMR devices are rapidly and more readily made with low-cost materials and without need for clean-room technology. We envisioned that flexible and transparent polymeric materials, like our PDMS device, was likely to enable uniform irradiation with low-power light sources to allow the development of new setups for photo-CIDNP on small-volume systems to tackle the concentration sensitivity limitations of such devices.

The CIDNP effect occurs as enhanced NMR signals in a target molecule that forms a radical pair (RP) with a photoexcited photosensitizer (commonly a flavin). Not all the nuclei of a molecule engaged in such an RP will experience the hyperpolarization phenomenon, but only those experiencing notable hyperfine interactions with the unpaired electron. This is important because, for historical reasons, the acronym DNP is contained within CIDNP, which might yield confusion. We recall that in solution DNP the enhancements can be experienced by all nuclei in a given molecule, as the dominant mechanism is the Overhauser transfer of the large electron spin polarization onto the nuclear spins; therefore, limiting the maximum theoretical enhancements to the electron/nucleus gyromagnetic ratio (~660 for proton). In CIDNP, the RP mechanism operates in a distinct way, where the enhancements are usually proportional to the hyperfine coupling constant and sometimes to its second or even third power in aromatic compounds. Two major traditional challenges of photo-CIDNP are lack of uniform irradiation of the whole sample volume and photodegradation of the sensitizer, e.g. flavin. Previous attempts to achieve a more homogeneous irradiation of the hundreds of microlitres contained within a conventional NMR tube dealt with more or less complex modifications of the optical fibre. Notably, flavin photodegradation is of high relevance because it limits the number of spectra that can be recorded on a sample. Using H$_2$O$_2$ to reoxidize the flavin comes at the risk of protein degradation or oxidation. Therefore, alternative approaches have been devised to minimize the impact of flavin degradation by enhancing the signal in shorter experiments, using complex pulse sequences to store and accumulate the polarizations, or their generation on heteronuclei ($^{13}$C or $^{15}$N) to be later detected on $^\text{1H}$ or $^\text{13}$C. More recently, the simultaneous use of three enzymes within the solution containing the target sample to reoxidize the flavin has been proposed. The use of LED-based illumination was recently devised to irradiate samples at lower power for conventional 5-mm NMR tubes. Nevertheless, the persistence of the bottleneck concerns, and the increasing complexity associated with each of the above improvements devised to surpass them, prompted us for a need of revisiting the method.

In the present manuscript we expand the field of applications of microcoils, showing their suitability to facilitate the performance of photo-CIDNP experiments with a number of advantages that we expect will rekindle the realm of these experiments, all this by virtue of the small active volumes afforded by these devices. The first clear advantage is that high-power light sources are no longer a prerequisite, because our setup requires irradiation of just one microlitre of sample. Moreover, the light source/ fibre is implemented in a non-invasive manner, not disturbing the sample detection volume. Finally, the setup allows for straightforward continuous flow photo-CIDNP experiments that, importantly, eliminates the necessity of light pulses, and even allows continuous irradiation. All this dramatically simplifies the experimental setup, with impressive mass and concentration sensitivity, saves measuring times because of elimination of dead/waiting times, and most importantly, resolves the problem of photodegradation products in the detection volume that results in signal loss. The experiments presented here are performed on a 9.4T magnet (400 MHz proton Larmor frequency), yet the home-built setup (Fig. 1) rivals cryoprobe sensitivity at higher fields.

Results

Initial remarks. Below, our results are organized as follows: first we describe how the setup as shown in Fig. 1 is simply made and assembled without clean-room technology or specialized equipment. Next, the performance of photo-CIDNP is evaluated using nucleotide and amino acid samples, followed by on-flow experiments monitoring supramolecular interactions as well as folding of polypeptides. Finally, sub-picomole limit-of-detection is presented.

System setup to perform photo-CIDNP experiments. The setup as shown in Fig. 1 to perform photo-CIDNP experiments was manufactured building on three main components, a light source placed outside the magnet, a microfluidic NMR chip, and an optical fibre to guide the light towards the active volume of the microcoil. To address uniform irradiation of the sample volume, both a current and a temperature controller are used to regulate the amount of output light from a blue laser diode ($\lambda = 455$ nm) operating in the mW range. The radiofrequency microcoils are incorporated within a polydimethylsiloxane (PDMS) crosslinked polymer defining a microfluidic device or NMR chip that is in turn embedded on a 3D-printed polyactic acid (PLA) holder. An important advantage of PDMS is that it is transparent in the visible light part of the electromagnetic spectrum, which facilitates an efficient light penetration. Accordingly, an optical fibre of 1 mm of internal diameter is inserted within the NMR chip holder, placed 2 mm above the solenoidal coil, to guide the light beam from the laser diode to the detection region of the microcoil containing the sample. This setup decreases heating effects of high-power irradiation and eliminates optical density limitations. High-power illumination (Watts, W) is a requirement in
conventional photo-CIDNP experiments to deliver as much light as possible into the hundreds of microlitres of sample inside the NMR tubes. We argue that it is possible to resort to more affordable, low-power lasers operating in the milliWatt (mW) range to perform photo-CIDNP experiments using PDMS-embedded microcoils to facilitate light penetration on the sample. To prove this concept, we used solenoidal microcoils with an active volume of just 1 μL within the framework of the setup presented.

We carried out photo-CIDNP investigations on the guanosine monophosphate nucleotide (GMP), since the H8 of the ring is reported to experience a signal enhancement upon light irradiation in the presence of sub-mM amounts of flavin mononucleotide (FMN)\(^40\). Accordingly, 20 mM (7.2 μg, 20 nmol) of GMP were irradiated at 180 mW during 1 s in the presence of 0.5 mM (0.9 μg, 0.5 nmol) of FMN. This resulted in the photo-CIDNP spectra shown in Fig. 2, which is a clear proof of concept of our setup to carry out photo-CIDNP experiments using microcoils. By measuring the signal-to-noise (SNR) ratio between the H8 signal in the light and dark spectra, a ~7-fold \(^1\)H photo-CIDNP enhancement is observed for GMP under these conditions.

**Photo-CIDNP microcoils under high optical densities.** To date, flavin concentrations in photo-CIDNP experiments were restricted to the sub-mM range (ca. 0.2 mM) due to their high optical densities in solution at higher concentrations\(^21, 27, 33, 34, 41, 42\). We argue here that increasing the concentration of flavins to the mM range, close to its aggregation limit (ca. 2 mM), would allow efficient photo-CIDNP by virtue of the small volumes afforded by microcoils alleviating the concentration restrictions.

To explore this hypothesis, we performed a series of photo-CIDNP experiments to study the intensity changes on the H8 signal of N-acetyl-L-tyrosine (AcTyr) in solutions containing different FMN concentrations of increasing optical densities; namely, 0.2, 0.5, 1.0, and 2.0 mM, while keeping constant the concentration of AcTyr to 5 mM. Analogous to our experiments on GMP described above, seven microlitres of each sample...
were injected into the microfluidic channel and irradiated at 180 mW during 6 s. The duration of the laser pulse was chosen according to literature precedents, where a light pulse of 6.4 s of duration yielded the maximum signal enhancement on photo-CIDNP experiments using 3-fluorotyrosine. This assumption was confirmed for our particular setup by optimizing the duration of the light pulse for our experimental conditions (Supplementary Figure 1). The resulting spectra confirmed our hypothesis that, despite of a prominent increase in optical densities when moving from 0.2 to 2.0 mM in flavin concentration, the signal enhancement obtained increases with an increasing concentration of photosensitizer molecules (Fig. 3). This proves that the small active volume located in a microfluidic channel of high surface-to-volume ratio is efficiently, uniformly irradiated using low-power sources regardless of the optical density of the sample. In fact, when moving from a normal NMR tube (Ø=5 mm) to a microchannel (Ø=650 μm), the Lambert-Beer law predicts an order of magnitude decrease in absorption at equimolar concentration, and hence the opportunity to increase the photosensitizer concentration accordingly. The (SNR) ratio corresponding to the H6 signal of AcTyr as measured from the dark and light spectra represents a ~6-fold 1H CIDNP enhancement under high optical density conditions.

Continuous flow photo-CIDNP experiments on microcoils. Having shown that the small active volumes afforded by microcoils allow precise, uniform light penetration to carry out photo-CIDNP experiments with low-power light sources, we next pursued overcoming the second and major challenge in photo-CIDNP; namely, to avoid the accumulation of photo-degraded flavin in the detection region in a simple yet efficient way. To this end, we exploited the versatility of customized microcoils to work under continuous flow conditions. This setup has significant advantages with respect to previous attempts reported in the literature to prevent flavin degradation29, 35, 43, since one can get rid of it by simply flushing continuous fresh sample through the active volume during irradiation.

To prove this concept, we recorded a series of photo-CIDNP spectra on 5 mM AcTyr in the presence of 1.0 mM FMN. Under static conditions, continuous irradiation during 12 min causes almost complete flavin degradation. This time of irradiation corresponds to acquisition of 8 consecutive spectra with 28 scans or accumulations each. Due to flavin degradation, a drastic decrease in the intensity of the H6 CIDNP signal of AcTyr is observed between the first and last spectra under these circumstances (Fig. 4a, left panel). Plotting the intensity of the CIDNP signal from each of the eight spectra shows its gradual decay over the course of the experiment (Fig. 4a, right panel). In a second experiment identically performed, but this time pumping fresh sample at a flow rate of 5 μL/min, the signal enhancement remained fairly constant for each of the eight consecutive photo-CIDNP spectra, as can be seen in Fig. 4b, where the first and last spectra show an equal polarization for the H6 protons of AcTyr. Accordingly, no intensity decay is observed when plotting the intensities of the H6 signals in the consecutive experiments (Fig. 4b, right panel), which confirms our hypothesis that working under continuous flow conditions fully prevents accumulation of degraded flavin within the detection region, even when the light source remains switched on during the whole experiment.

The observation that the SNR of the AcTyr CIDNP signal is better under continuous flow conditions (Fig. 4b) with respect to the static experiments (Fig. 4a) is the result of two distinct effects enabled by flow conditions. On the one hand, it is reported that flow intrinsically increases the SNR with respect to static conditions as it ensures pre-equilibration of all sample nuclei and decreases the effective relaxation time44. On the other hand, each of the experiments shown in Fig. 4 consists of 28 scans, a time during which degraded flavin is already accumulating within the active volume that is subjected to continuous irradiation. Therefore, under static conditions signal averaging within each experiment comprises increasing amounts of degraded flavin that does not contribute to the CIDNP signal, which is not the case under continuous flow conditions where the sample located within the active volume is continuously refreshed. However, one
should bear in mind that static conditions use just 1 μL of material, a much lower amount than that required for flow conditions. Nevertheless, only 60 μL of sample (67 μg of AcTyr) were required to record this large number (>200) of 1D spectra under flow conditions. Considering that conventional photo-CIDNP experiments employ light pulses alternated with dark periods of several seconds (usually >15 s, see Supplementary Figure 2) for pulsed irradiation on microcoils), the use of continuous irradiation under continuous flow conditions results in much shorter experiment times with much lower amounts of sample.

Efficient mixing by on-flow photo-CIDNP on microcoils. A key feature of photo-CIDNP experiments is the unique capability to non-invasively monitor solvent accessibility of aromatic residues in peptides and proteins21, 27, 49–50, whose signal intensities or chemical shifts are modified according to changes in their solvent exposure or burial. Accordingly, we tested our system’s ability to detect such changes by monitoring the Hε resonance of the Tyr moiety in two different scenarios; namely, (i) when the amino acid becomes less exposed to the solvent using an encapsulating agent, and (ii) when it becomes more exposed to the solvent using a denaturing agent in the context of a wellfolded peptide. For the first experiment we studied the encapsulation of AcTyr by β-cyclodextrin (β-CyD). In the second experiment we followed changes of two tyrosine residues in the 197-TGWKKIADKWYYFN-210 sequence, which corresponds to the pneumococcal autolysin protein LytA (LytA239–252), in the presence and absence of 4.5 M urea.

In order to carry out these studies under continuous flow conditions that would benefit from all the advantages exposed in the previous section, we further optimized the NMR chip device with a new design that contains two inlets (two entries for sample, see Fig. 1). The idea beneath this design consists of enabling rapid and efficient mixing of the two samples prior to irradiation and detection, so that conventional elaborated setups37, 38 can be avoided. Supplementary Figure 3 proves the quality of the mixing by fluorescence microscopy. The system setup consists of two syringes, a first one containing 2 mM FMN and the target sample (AcTyr or LytA239–252), and a second one with β-CyD or urea, respectively, to promote solvent burial or exposure of the corresponding target molecule. A flow rate of 2.5 μL/min was used to load each sample through the corresponding inlet, so as to have a net entry of 3 μL/min, according to the flow conditions used in the previous section.

Figure 5a shows the photo-CIDNP spectra corresponding to the outcome of the first experiment (mixing of AcTyr and β-CyD to promote solvent burial). When the syringe containing β-CyD is switched on, both β-CyD and AcTyr are in contact, which results in the encapsulation of the amino acid. Accordingly, the emissive signals of its Hε protons slightly shift, broaden, and lose intensity as a consequence of the supramolecular interaction that lowers the solvent accessibility of AcTyr (see also Supplementary Figure 4). On the other hand, when the syringe pump containing β-CyD is switched off, the resulting photo-CIDNP spectra is identical to that shown in Fig. 4 for freely, solvent-exposed AcTyr. This behaviour is consistently reproduced.

In the second experiment (mixing of LytA239–252 and urea to promote solvent exposure), switching on the syringe containing urea allows mixing of urea and LytA239–252. Therefore, the high concentration of denaturizing agent causes a loss of native structure that results in unfolded conformations in which the two tyrosines are solvent exposed. Consequently, two peaks corresponding to the Hε protons of two tyrosines appear in the photo-CIDNP spectra under these circumstances (Fig. 5b). Although there is no deposited structure for this system in the Protein Data Bank, the original paper reporting the NMR solution structure of LytA239–252 clearly illustrates the two tyrosines as having two radically different atomic environments52: Tyr249 (Y249) is clustered against side chains from F251, I244, and K247, establishing a number of intermolecular contacts. In contrast, Tyr250 (Y250) is highly exposed to the solvent. These reported data reconcile our observation of one and two tyrosine signals when urea is absent and present, respectively.

Microcoils and photo-CIDNP enable sub-picomole detection. To explore the limit-of-detection that can be achieved by combining the enhanced mass sensitivity of microcoils with photo-CIDNP, we bear in mind that CIDNP intensities are directly related to the magnitude of the hyperfine couplings in the corresponding transient radicals24, 25. In this regard, 19F photo-CIDNP NMR studies of fluorinated compounds are very convenient for two reasons; namely, the natural abundance of 19F (100%), and the unusually large hyperfine coupling constant of this nuclide in p-fluorophenol53–55. This prompted us to prepare solutions of different concentrations and make dilutions until we could not detect any 19F CIDNP signal upon irradiation. We reached this limit at a sample concentration of 0.8 μM, which considering the 1 μL active volume corresponds to sub-picomole amount of material. Figure 6 (top) shows the dark spectrum of a sample containing 0.4 mM of p-fluorophenol
Dangling bonds on the heater and the copper coil, which can be detected. Figure 6 (bottom) shows the corresponding 19F NMR spectrum of a sample containing 0.4 mM of p-fluorophenol, which cannot be detected. The 19F NMR spectrum under flow conditions of a 0.8 μM p-fluorophenol sample. This corresponds to detection of just 0.8 picomole of material per μL. The number of accumulated scans is 14 in both cases.

Photo-CIDNP experiments. 1H photo-CIDNP experiments on microcoils were carried out in a 9.4T narrow-bore Oxford instruments magnet (400 MHz 1H Larmor frequency and 376 MHz 19F Larmor frequency) INOVA NMR spectroscopy (Oxford, Agilent). A commercial probe was used as the scaffold onto which the hand-made solenoids embedded in the PDMS matrix were mounted. The procedure to customize these microcoils is described in detail elsewhere. The low-power light source used for the experiments consists of a laser diode emitting at 450 nm, operating at 1.6 W output power. The optical power is lowered to the milliWatt range by using a potentiometer that regulates the current through the diode, and a photometer was used to monitor the desired power from the light beam coming out of a 1 mm (internal diameter) optical fibre. This current controller (Thorlabs, LDC 205C/240C) can be switched on and off to regulate the duration and intensity of the light pulse manually (continuous irradiation) or by a TTL signal directly from the spectrometer, therefore to be controlled by the user through the pulse sequence, for light pulses photo-CIDNP experiments. A temperature controller (Thorlabs, TED 200C) is also used to ensure no variation of the output light power.

For experiments performed under flow conditions, a total flow rate of 5 μL/min is created using syringe pumps as depicted in Fig. 1. The flow rate is used to carry out these experiments. In practice, typically a 100 μL glass syringe is used, and the flow is handled using syringe pumps.

All the samples (GMP, FMN, AcTyr, and β-CyD) were purchased from Sigma Aldrich and directly dissolved in 100% D2O to the concentrations specified for each experiment. The peptide corresponding to Lys129-131 was obtained from R-Peptide, whose identity and purity (>95%) were confirmed by HPLC and mass spectrometry. Spectra were processed in Vnmrj, and all signal-to-noise ratios (SNRs) were determined as the maximum height of the peak divided by the root-mean-square noise of the baseline using the SNR peak calculator script implemented in MestReNova (http://www.mestecnova.com).

Data availability. All relevant data supporting the results of this study are available from the corresponding author upon request.

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Fig. 6 Sub-picomole detection combining photo-CIDNP and microcoil NMR technology. Top: 19F NMR spectrum of a sample containing 0.4 mM of p-fluorophenol, which cannot be detected. Bottom of figure: The photo-CIDNP spectrum under continuous irradiation. The situation is drastically different when the sample is irradiated in the presence of equimolar amounts of flavin, to such extent that even a small sample containing 0.8 μM of p-fluorophenol could be detected. Figure 6 (bottom) shows the corresponding 19F photo-CIDNP spectrum, where it is possible to detect such a diluted sample that corresponds to just 0.8 pmol of material per μL.

Discussion

An application of microcoil technology that boosts NMR mass and concentration sensitivity by combining these devices with a hyperpolarization scheme, photo-CIDNP, is presented. Performing these experiments on reduced-diameter NMR coils smartly overcomes traditional challenges of the technique; namely, high-power irradiation, lack of uniform irradiation, and accumulation of degraded flavin in the detection region. The combination of a PDMS matrix with low-power light sources employed in our setup alleviates heating effects, especially in light of the low heat-conductivity properties of PDMS. It is remarkable that the use of LED-based illumination was recently devised to irradiate samples at lower power for conventional 5-mm NMR tubes, as discussed in the introduction. In our setup, we have shown that the small active volumes and the possibility to work under continuous flow conditions prevent these challenges, all within the framework of a very simple setup in which the microcoils employed are easily manufactured within only one day, and just using basic lab equipment and materials. Furthermore, the combination of continuous flow conditions and low-power irradiation allows working with continuous irradiation, which removes the need to trigger light pulses from the spectrometer, reducing experiment time, and to resort to complex pulse sequences.

In summary, we present the development of a simple, powerful and low-cost setup to perform photo-CIDNP with high efficacy on 1 μL detection volumes and with uniform illumination of the entire sample and avoiding high-power laser that overcomes current limitations in the field. This makes photo-CIDNP more accessible to the scientific community and may open new opportunities based on a rapid, efficient mixing of samples to develop novel applications. Current possibilities under investigation by virtue of the possibilities of the setup presented here, include expanding LODs to the sub-picomole level, along with the possibility of performing 2D heteronuclear experiments owing to our recently developed broadband, non-resonant microcoils with active volumes in the nanolitre range.

Methods

Characterization of the transceiver solenoidal NMR microcoil. The solenoidal coil manufactured to perform the experiments presented in this work consists of a coated copper wire (100 μm diameter) encompassing a cylindrical volume with Ø = 650 μm. The resulting coil has a total length of 3 mm, and covers a detection volume of 1 μL. The microcoil is mounted on the probe and electrically connected to three capacitors that allow tuning and matching to 1H and 19F frequencies, similar to the setup described by Popovic and coworkers, as illustrated in Fig. 1 (see magnified region). Aulation curve is shown in Supplementary Figure 5 to highlight the excellent B1 field homogeneity of the coil.

To characterize the performance of this transceiver microcoil, we have used the mass and concentration sensitivity (Sm and Ss, respectively) and the normalized (mass and concentration) limit-of-detection (nLODm and nLODc) parameters, which are appropriate indicators for mass-limited samples and to compare microcoil probes of different active volumes and whose definitions can be found in ref. 8. Using a sample consisting of 20 mM GMP in 100% D2O, the following values were obtained from a single-scan experiment with an acquisition time of 0.5 s: Sm = 1433 (2790) SNR μmol−1 s−1/2, Ss = 1433 (0.014) SNR μmol−1 s−1/2, nLODm = 2 (55) nmol s−1/2 SNR−1, and nLODc = 2 (0.27) mM s−1/2 SNR−1. The values in parenthesis correspond to the 5-μL solenoidal microprobe reported by Olson et al., and have been already scaled up to 400 MHz for a proper comparison using the B3LYP factor. As expected, the smaller the active volume, the higher the mass sensitivity, and the lower the concentration sensitivity.

Additional references include expanding LODs to the sub-picomole level, along with the possibility of performing 2D heteronuclear experiments owing to our recently developed broadband, non-resonant microcoils with active volumes in the nanolitre range.
the manuscript; V.S. and R.M.S.-D. fabricated solenoidal microcoils; M.V.G., A.H.V., A.d.l.H. and M.M. discussed the data. A.d.l.H. facilitated the performance of the project.

**Additional information**

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