Emerging Life (EmLife): Technology for the High-Throughput Analysis of the Molecular Composition of Small Volumes

Denis Morsa,[1] Emily Kempa,[1,2] Iuliia Stroganova[1], Aigars Piruska[3], Wilhelm T.S. Huck[3], Philippe Nghe[4], Eörs Szathmáry[5], Wim Hordijk[5], Jan Commandeur[6], Sijbren Otto[7], Anouk M. Rijs[1*]

[1] Radboud University, Institute for Molecules and Materials, FELIX Laboratory, Toernooiveld 7, 6525 ED Nijmegen, The Netherlands
[2] Michael Barber Centre for Collaborative Mass Spectrometry, Manchester Institute of Biotechnology, Manchester M1 7DN, UK
[3] Institute for Molecules and Materials, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands
[4] École Supérieure de Physique et Chimie Industrielles de la Ville de Paris (ESPCI Paris), Paris Sciences Lettres University, France
[5] Parmenides Center for the Conceptual Foundations of Science, Parmenides Foundation, Pullach, Germany
[6] MS Vision, Televisieweg 40, 1322 AM Almere, The Netherlands
[7] Centre for Systems Chemistry, Stratingh Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

*Corresponding author: * a.rijs@science.ru.nl

ABSTRACT
The high-throughput characterization of the molecular composition of small sample volumes is an unsolved problem. Solutions to this problem would be game changing to fields ranging from origins of life research to diagnostics. Objective of the EmLife consortium is to develop a solution based on interfacing microdroplet technology with mass spectrometry. The new technology will initially be validated and utilized for addressing a grand challenge in fundamental science: the origin of life. However, the new technology has the potential to revolutionize not only fundamental science, but also detection methods in medicine. Specifically, partners will be sought to develop applications in diagnostics.

Keywords: High-Throughput, Small Volume Sampling, Droplet-Microfluidics, Mass Spectrometry, Origin of Life

1. INTRODUCTION

Determining the molecular composition of small volumes (picoliter [=10^{-12} liter] range) in high-throughput fashion is currently impossible. Yet microfluidics technology exists for handling small sample volumes and mass spectrometers exist that can rapidly analyse the molecular composition of complex mixtures. Successfully interfacing these technologies would be a game changer in many field ranging from fundamental science to applications in health care. The goal of the EmLife consortium is to develop new technology for high-throughput analysis of complex mixture contained in microscopic volumes. This effort is driven by a community of leading scientists from investigating the origin of life combined with experts on mass spectrometry. Beside the origin of life, this development has the potential to revolutionize the way diagnostics is done in health care.

A major challenge in origin of life research is to get autocatalytic, self-reproducing molecular reaction networks to emerge and evolve in protocells, *i.e.*, small compartments. Autocatalytic networks have been studied extensively both theoretically and experimentally. However, so far nobody has been able to let them emerge spontaneously from basic starting material, and then evolve and diversify further in small compartments. A highly promising approach to this challenge is the use of droplet microfluidics where small aqueous droplets are suspended in an oil solution. However, implementing this approach would require new technology for automated, high-throughput analysis of molecular compositions in small volumes.

We have developed a prototype of such integrated technology, based on coupling droplet microfluidics technology with mass-spectrometry technology, see Fig.1. Our results show that microdroplets microfluidics can be interfaced with mass spectrometry. We have investigated the best working experimental conditions and instrumental parameters, followed by a preliminary exploration of its use for self-replicating systems.

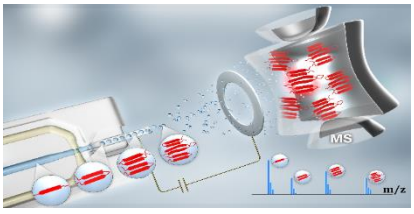


Fig.1: Schematic representation of the developed technology showing the droplet-microfluidics with integrated MS emitter on the left and the MS collection and detection on the right

2. STATE OF THE ART

Mass spectrometry is the most powerful tool for identifying the molecular composition of complex

samples, due to its high sensitivity -allowing to detect and identify a nanogram of protein which resembles 1 grain of sugar in a cubic kilometre of water in targeted analysis- and the ability to distinguishing molecules based on their molecular weight. Droplet-microfluidics technology is the state-of-the art method for handing small (picoliter-sized) samples in high throughput (up to 10,000 droplets per second). Thus, it appears obvious to combine these two techniques, yet surprisingly little effort has been made to develop such a combination. Early contributions have described prototype microfluidic chips that were interfaced with commercial mass spectrometers.[1,2] Results were promising, but these devices were limited in throughput and sensitivity and not further developed. Earlier this year some of us reported methodology that not only improved throughput but also the ease of interfacing with existing mass spectrometers.[3] The work presented herein builds on this state-of-the art technology.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

When it comes to high-throughput analysis of the molecular composition of small sample volumes, no other technique comes close to our microdroplet – mass spectrometry approach. In the past microfluidics has been coupled to other instrumentation capable of detecting features of molecules, but these almost invariably rely on spectroscopic features (UV, Raman, fluorescence, NMR, etc.). These approaches are the methods of choice for samples in which only one or a few types of molecules are present. However, when complex mixtures (such as prebiotic reaction networks, or bodily fluids) are to be analysed, the output of these methods becomes very difficult to disentangle. Mass spectrometry has the unique advantage over these other methods in providing a separate signal for each type of molecule that is readily identifiable and quantifiable.

4. PROJECT RESULTS

Microdroplets were produced using a chip made of polydimethylsiloxane (PDMS) and comprising two inlets, one outlet and a network of connecting channels in between (Fig.2). Droplets are created via flow-focusing which consists in pushing a stream of aqueous analyte sample into a continuous phase oil stream. The size and volume of the droplets are typically controlled by the geometry of the chip, i.e. the channel heights, and flow rates of the aqueous (Q_{dis}) and oil (Q_{con}) streams. In practice, the process may be monitored under a microscope (Fig.2c). Interfacing microdroplets microfluidics with MS was achieved by modifying the commercial ESI probe furnished by the instrument manufacturer (Bruker, Bremen, Germany).

Different strategies may be envisioned when it comes to using microdroplets: (i) direct injection, (ii) offline droplets collection and subsequent reinjection and (iii) online droplets trap-and-release.[1-4] The first strategy relies on a flow-focusing junction and allows for direct sampling of the analyte content, similarly to direct-infusion ESI. The two other strategies allow to store the droplets, either offline or on-the-chip, so that their content can mature before analysing it.

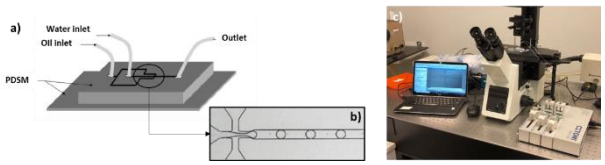


Fig. 2. Schematic representation of the droplet-microfluidics assembly with (a) PDMS chip (LxWxH=20x8x5mm, with 130mm emitter with inner diameter of 75 µm), (b) flow-focusing microdroplets generation resulting in picoliter droplets and (c) microscope with computer-controlled syringes.

4.1 Droplet-microfluidics-MS interface

For the proof-of-principle experiments on the interface of droplet microfluidics with the Bruker ion trap mass spectrometer, a set of characterization experiments have been performed. The direct injection microfluidics chips have been used (i-iii) as well as the collection and reinjection chips (iv):

(i) Detection limits

Limits of detection (LOD) were determined as 150 µM for small ions (m = 200 Da) and 250 µM for proteins (m = 8 kDa). This is currently limited by the type of mass spectrometer used rather than the developed droplet-microfluids device. This will be significantly improved by the use of a time-of-flight mass spectrometer in the follow-up experiments.

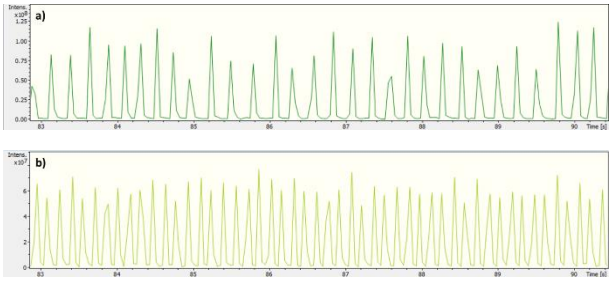


Fig. 3. Total ion current (TIC) chromatograms during a microdroplet-MS experiments. (a) Q_{dis}/Q_{con} = 0.2, signal ~4 Hz and (b) Q_{dis}/Q_{con} = 0.5, signal ~6.6 Hz.

(ii) Influence of flow rates on droplet frequency

Fig.3 illustrates the typical evolution of the total ion current chromatograms during a droplet-microfluidics MS experiment on the pentapeptide Leu-Enk (c= 100 µM). One pulse corresponds to the signal achieved over

one droplet of dispersed phase (Q_{dis}), the valley between the pulses corresponds to the condensed phase (Q_{con}) between two adjacent droplets. The pulse frequency is correlated to the droplet frequency and may be adjusted via the Q_{dis}/Q_{con} ratio, resulting in a droplet frequency of (a) 4 Hz with Q_{dis} = 20 µL/h and Q_{con} = 100 µL/h for the top trace and (b) ~6.6 Hz with Q_{dis} = 50 µL/h and Q_{con} = 100 µL/h for the bottom trace. The volume of a droplet is ~2000 pL.

(iii) MS signal over accumulated droplets

Fig.4 shows the MS spectra of the dipeptide diphenylalanine (FF, c = 400 µM) after accumulation over (a) 100 droplets, (b) 10 droplets and (c) 1 droplet. The monomer $[M+H]^+$, dimer $[2M+H]^+$ and to a certain extent trimer $[3M+H]^+$ are visible. Additional peaks at the higher mass range are related to the surfactant used for droplets production and stabilization.

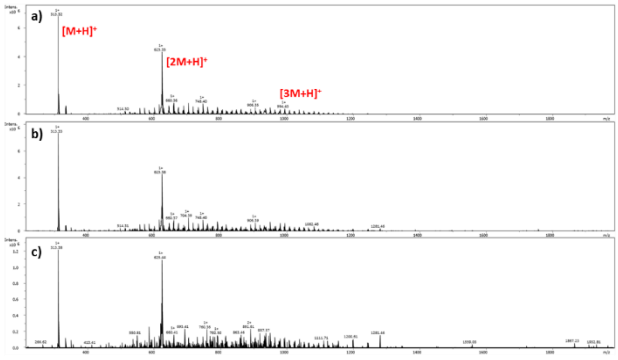


Fig. 4. MS spectra of the FF dipeptide (c = 400 µM) after accumulation over (a) 100 droplets, (b) 10 droplets and (c) 1 droplet. Q_{dis} = 50 µL/h and Q_{con} = 100 µL/h.

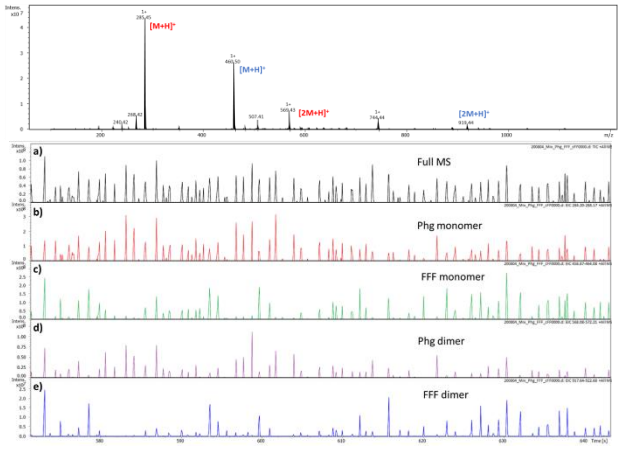


Fig. 5. Top: MS spectrum of mixed droplet collections of FFF (blue) and Phg (red). **Bottom:** chromatograms showing the (a) total mix, individual droplets containing (b) $[M+H]^+$ of Phg, (c) $[M+H]^+$ of FFF, (d) $[2M+H]^+$ of Phg, (e) $[2M+H]^+$ of FFF.

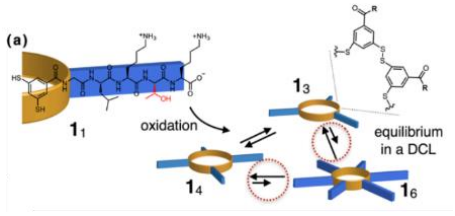
(iv) Identification of mixed collections

As a proof of concept, fractions of peptides (c = 400 µM), triphenylalanine (FFF, m=460 Da) and diphenylglycine (Phg, m=285 Da) were collected, pooled and reinjected using microfluidics ESI. The following parameters were used for collection: Q_{dis}= 100 µL/h and Q_{con}= 200 µL/h and for reinjection Q_{dis}= 20 µL/h and Q_{con}= 100 µL/h. Fig.5 (top) shows the mass spectrum summed over 10 droplets, with peaks corresponding to the monomer and dimer of both FFF (blue) and Phg (red). Fig. 5 (bottom panels) shows how the TIC chromatogram traces are correlated: traces of monomer and dimer belonging to the same peptide have the same modulation, as they originate from the same droplets. However, these are different from the traces belonging to the monomer and dimer of the other peptide which are encapsulated in different droplets.

4.2 Application to self-replicating systems

Having established that our microdroplet-MS technology is capable of correctly identifying the compositions of "trivial" microdroplet samples (down to micromolar concentrations at rates up to 6 droplets per second), we extended our work to a small molecular network that, with time, gives rise to self-replicators. Building block **1**$_1$ was allowed to oligomerize, initially producing timers **1**$_3$ and tetramers **1**$_4$ that do not replicate and eventually transforming into self-replicating hexamer **1**$_6$ (Scheme 1).[5]

Preliminary data on this system is encouraging. ESI MS spectra (Fig. 6) are dominated by the trimer and tetramer intermediates, while the peak at ~1515 Da is associated with the hexamer replicator. While some optimization is needed, these results open the way for using microdroplet technology for evolving systems of synthetic self-replicators for the first time.



Scheme 1. Oligomerisation of building block **1**$_1$ gives rise to a dynamic combinatorial library of differently sized macrocycles from which the hexamer **1**$_6$ is able to self-replicate.[5]

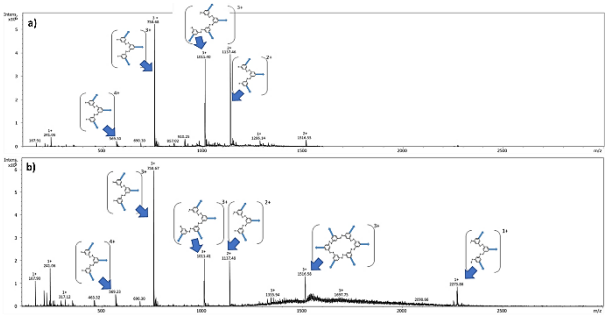


Fig. 6. ESI-MS spectra of the combinatory systems for target masses of (a) 1200 Da and (b) 2000 Da.

5. FUTURE PROJECT VISION

In the present work, we developed an interface between microdroplets microfluidics and MS, investigated the best working experimental conditions and instrumental parameters, followed by a preliminary exploration of its use for self-replicating systems. From a technical point of view, the microdroplets microfluidics interface provides an exploitable MS signal that is on par with the signal achieved by direct-infusion ESI when considering a same accumulated timeframe. As an advantage, less salt adducts were formed when using microfluidics. As a drawback, surfactant peaks are present due to the continuous phase. These results, together with those we reported previously[3], provide proof of principle and show that the basic technology works. They also provide a strong impetus to develop the technology further into a valuable tool capable of addressing wide-ranging applications.

The following steps are essential to bring the product forward:

- Improving the sensitivity such that molecules can be detected at 1000-fold lower concentration (nanomolar and ideally below).
- Improving the throughput by a factor 10-100.
- Incorporating sample treatment options in the microfluidic devices, such that conditions in individual sample droplets may be altered to improve the ionisation in the mass spectrometer.
- Minimising the effect of the continuous phase and the associated surfactants on detection efficiency.
- Parallel to these important technological improvements the new technology will be employed for the development and study of autocatalytic networks with the aim of achieving Darwinian evolution. Success would be an excellent showcase of the power of the new technology.

5.1. Technology Scaling

The microdroplets microfluidics MS interface is now at TRL3, as demonstrated by the experimental proof of concept described here. In order to take it to TRL4 it will be necessary to validate the technology further by establishing the sensitivity, dynamic range and reproducibility that can be achieved. Incorporation of sample treatment capabilities, including those aimed at enhancing the efficiency of the ESI process, whilst minimising the effect of the continuous phase, must be characterised and optimised. In the process, key applications for the interface can be identified or confirmed based on the results obtained. Validation of the technology for such applications on real-life samples will take it to the next TRL. Specific case studies in these applications, now foreseen in the areas of autocatalytic sets and medical diagnostics, will ensure extensive use of the interface in various configurations. Successful completion of these studies brings the technology to TRL6 and provide the practical experience and feedback required to engineer a prototype that can be sold to early adopters on a variety of different MS platforms. In close cooperation with these early adopters, all aspects of the system will be integrated further, including software and compatibility with many MS platforms, yielding a TRL of 7.

5.2. Project Synergies and Outreach

To provide the expertise and resources required to take the technology to TRL 7, more partners will be added to the consortium: The group of Prof. Rijs, expert on hyphenated mass spectrometry and spectroscopy of peptides assembly, who was already included during the first phase, will continue to improve the connection between the droplet-microfluidics and mass spectrometry. Additionally, prof. Barran (University of Manchester) develops mass spectrometry based methods and instruments for the analysis of biomarkers for Parkinson's Disease from sebum[6, 7] will be part of the consortium. The group is pioneering the development of alternative sources for MS, amongst them the coupling microdroplets and MS.[3] MS Vision is an SME that is specialised in independent MS support and modification. It will be their responsibility to translate the specific technology requirements into a flexible and robust design, and eventually bring the technology to the market.

During Phase 1 we engaged with "Research Outreach" resulting in a 4-page popular science article about our ATTRACT project which appeared online and in print, followed by a social media outreach campaign. We envision working with this organisation again during Phase 2 to produce additional articles, and also one or more (animated) movies about the project. Furthermore, we will write several popular science articles ourselves, and present public lectures about our research project. During Phase 1 we also created a dedicated project website (https://coolscience.club/), which we will continue to use and expand.

5.3. Technology application/demonstration cases

The consortium will focus on the application and demonstration cases:

(1) Droplet microfluidic for the origins of life

Autocatalysis is at the core of metabolism, reproduction and evolution in living systems. The team has laid out a roadmap to go from existing autocatalytic systems to life.[8] The team also developed breakthrough mathematical models and computational tools for the detection of new autocatalytic systems. These tools coupled with MS and droplet microfluidics offer the first generic method to screen for autocatalytic systems. The droplet-MS interface is an enabler for monitoring their subsequent evolution.

Autocatalysis also has industrial applications, among which: (i) enantiomer amplification for pharmaceuticals;

(ii) exponential chemical amplification for the detection of pollutants or harmful compounds; (iii) high resolution photolithography. Furthermore, harnessing artificial self-replication will allow us to create artificial biomimetic chemistry including self-healing, highly responsive materials exhibiting exponential growth, and adaptive systems. This is to achieve TRL6: demonstrate that the technology provides answers to real-life questions.

(2) Droplet microfluidic for health care diagnostics

Additional technological and societal utilization can be found in the application of the droplet microfluidics - MS technology for biomarker discovery. Biomarker applications are related to the characterization of individual molecules in a heterogeneous sample. Success of the method and the unambiguous identification of metabolites, i.e. small "disease reporter" molecules, requires high selectivity, sensitivity and efficiency (high-throughput) of the chosen analytical approach. These requirements will be met by our Microfluidics-MS technology. For the application and demonstration, we will first concentrate on the screening of disease biomarkers for Parkinson's Disease by including body-fluids in the droplets. The focus lies on developing the technology as a high-throughput screening platform with minimal sample/reagent volumes and high analysis speeds, combined with high information content: all required to bring our technology from the lab into the clinic.

5.4 Technology commercialization

Currently no commercial package is available, either as an MS instrument option or as a field upgrade, to provide the functionality that our technology will offer. Early on in the commercialization process, we will reach out to key opinionmakers in the scientific community, promote the technology to them and seek cooperation on specific applications. Eventually these opinionmakers may turn into early adopters, that will themselves promote the product by publishing their results, and provide first-hand user feedback for further product development. Continuous dialogue with the scientific community and R&D departments of industry will help us finetune the market niches to address. Once these have been clearly identified, these will be targeted, aided by specific publications and product presentations at strategically selected conferences. The finished product will be compatible with the most popular MS models, irrespective of manufacturer. This ensures maximum market size. We will seek to open discussions with MS manufacturers on integration of the product in their standard MS instrument offering.

5.5 Envisioned risks

The technology at the heart of this project is based on prior art that is available in the public domain, and does not currently contain any aspect that can be protected through patents. At the same time, this makes it very unlikely that prohibitive IP can be created by others. During further development of the product, we will actively check project results for potential IP protection.

5.6 Liaison with Student Teams and Socio-Economic Study

Teams of MSc student, recruited through advertisements at leading universities and through the extensive network of the PIs associated with the project, will assist in the development of two different aspects of our new technology: (a) the design and simulation of new microfluidic devices (led by our Paris and Manchester hubs) and (b) the development of software tools to analyse the extensive MS datasets in search for new autocatalytic sets (led by Hordijk and MSVision). A summer school at the CERN IdeaSquare provides an excellent setting for intense and focussed efforts on these two topics.

The EmLife consortium will provide all requested information to assist in the socio-economic evaluation of the ATTRACT initiative. Requests will be handled by the coordinator who will solicit input form the other members of the consortium, where appropriate, and material and data from which impact of our technology can be assessed will be made available.

6 ACKNOWLEDGEMENT

This project has received funding from the ATTRACT project funded by the EC under Grant Agreement 777222.

7. REFERENCES

[1] Fidalgo, L. M.; Whyte, G.; Ruotolo, B. T.; Benesch, J. L. P.; Stengel, F.; Abell, C.; Robinson, C. V.; Huck, W. T. S. Coupling Microdroplet Microreactors with Mass Spectrometry: Reading the Contents of Single Droplets Online. *Angew. Chem. Int. Ed.* **2009**, *48*, 3665–3668.

[2] Smith, C. A.; Li, X.; Mize, T. H.; Sharpe, T. D.; Graziani, E. I.; Abell, C.; Huck, W. T. S. Sensitive, High Throughput Detection of Proteins in Individual, Surfactant-Stabilized Picoliter Droplets Using Nanoelectrospray Ionization, *Anal. Chem.* **2013**, *85*, 3812–3816.

[3] Kempa, E.E.; Smith, C. A.; Li, X.; Bellina, B.; Richardson, K.; Pringle, S.; Galman, J. L.; Turner, N. J.; Barran, P. E. Coupling Droplet Microfluidics with Mass Spectrometry for Ultrahigh-Throughput Analysis of Complex Mixtures up to and above 30 Hz. *DOI: 10.1021/acs.analchem.0c02632.*

[4] Toprakcioglu, Z.;Kumar Challa, P.; Levin, A.; J. Knowles, T. P. J. Observation of molecular self-assembly events in massively parallel microdroplet arrays, *Lab Chip*, **2018**, *18*, 3303–3309.

[5] Carnall, J. M. A.; Waudby, C. A.; Belenguer, A. M.; Stuart, M. C. A.; Peyralans, J. J.-P. Otto, S. Mechanosensitive self-replication driven by self-organization. *Science* **2010**, *327*, 1502-1506.

[6] Sinclair, E.; Trivedi, D. K.; Sarkar, D.; Walton-Doyle, C.; Milne, J.; Kunath, T.; Rijs, A. M.; de Bie, R.; Goodacre, R.; Silverdale M.; Barran, P. Sebum: A window into dysregulation of lipid metabolism in Parkinson's Disease, *ChemRxiv* **2020**, *https://doi.org/10.26434/chemrxiv.11603613.v2*.

[7] Sinclair, E.; Walton-Doyle, C.; Sarkar, D.; Hollywood, K. A.; Milne, J.; Lim, S. H.; Kunath, T.; Rijs, A. M.; de Bie, R.; Silverdale, M.; Trivedi, D. K.; Barran, P. Validating differential volatilome profiles in Parkinson's Disease, *ChemRxiv* **2020**, *https://doi.org/10.26434/chemrxiv.12525323.v1*.

[8] Adamski, P.; Eleveld, M.; Sood, A.; Kun, Á.; Szilágyi, A.; Czárán, T.; Szathmáry, E.; Otto, S. From self-replication to replicator systems en route to de novo life, *Nat. Rev. Chem.* **2020**, *4*, 386–403.