Photoluminescence-Based Techniques for the Detection of Micro- and Nanoplastics

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Dedicated to Professor Vincenzo Balzani in the occasion of his 85th birthday
Abstract: The growing numbers related to plastic pollution are impressive, with ca. 70% of produced plastic (>350 tonnes/year) being indiscriminately wasted in the environment. The most dangerous forms of plastic pollution for biota and human health are micro- and nano-plastics (MNPs), which are ubiquitous and more bioavailable. Their elimination is extremely difficult, but the first challenge is their detection since existing protocols are unsatisfactory for microplastics and mostly absent for nanoplastics. After a discussion of the state of the art for MNPs detection, we specifically revise the techniques based on photoluminescence that represent very promising solutions for this problem. In this context, Nile Red staining is the most used strategy and we show here its pros and limitations, but we also discuss other more recent approaches, such as the use of fluorogenic probes based on perylene-bisimide and on fluorogenic hyaluronan nanogels, with the added values of biocompatibility and water solubility.

1. Introduction

Sensitization campaigns on environmental concerns are exponentially growing but pervasive scientific ignorance and collective selfishness slow down the necessary global awareness to guarantee environmental sustainability of human activities. In this context, Professor Vincenzo Balzani is a strong voice, based on scientific evidence and data, in favour of a radical change in energy production and behaviour, of a transition toward a circular economy as our only chance against the terrible ecological and social crisis that we are living. Meanwhile, more and more severe problems are emerging like the ubiquitous presence of micro- and nano-plastics (MNPs). These particles are the most dangerous form of plastic pollution for biota and human health since they were found everywhere – in marine and freshwater ecosystems, sediments, soil and air –, they are more easily bioavailable – ingested by mammals, birds and fishes and found also in deep ocean creatures – and through the food chain they reach humans. Their detection and elimination is therefore a great, difficult challenge for the present and next future.[2–14]

We are all well aware that plastic has a central role in contemporary society but numbers are impressive (Figure 1): the global production of plastic has reached more than 350 million tons per year[15] and the fate of only circa one-third of it is known – recycled (9%), used for energy recovery (12%) and discard in landfills (8%)[15] – while more than two-thirds accumulate – and persist for a long time – in the environment through accidental release and indiscriminate waste. The latest estimates of plastic pollution are appalling and the fraction released into rivers and oceans is predominant[15] daily circa eight millions of pieces of plastic reach the ocean that means that yearly between 4.8 and 12.7 million tonnes litter them.[16] It is important to underline that plastic is a general term that includes a variety of different polymeric materials often added with diverse species to improve their performance or reduce their cost.

However, most plastics are light, durable and water insoluble, consequently hardly degradable even through weathering and aging. As mentioned above, only in recent years scientists are gaining more and more awareness of the danger and impact of their fragmentation in small pieces to produce MNPs.[17,18] Their origin, however, is not only disintegration (secondary micro- and nanoplastics) but they can also be intentionally...

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Figure 1. Global plastic production and global trends in millions of tonnes. Image available at https://www.grida.no/resources/6923. Year 2018. Credit: Maphoto/Riccardo Pravettoni. Data source: M. Bergmann, L. Gutow, M. Klage (eds) Marine Anthropogenic Litter (2015) Springer.
produced (primary micro- and nano-plastics), such as the beads used in cosmetics and personal hygiene, or in the industrial sector as precursors of plastic products. It is therefore of utmost importance to regulate their production but also to understand their generation to evaluate size, distribution, and surface chemistry, and thus hypothesize their interactions and their possible impact on different biota. Both direct and indirect toxic effects of micro- and nanoplastics, in fact, are very difficult to be evaluated and far to be understood. Their high bioavailability greatly increases the chances of hazard for human health, with exposure occurring not only by ingestion but also by inhalation, or absorption by the skin. Together with the toxicity rising from their composition and dimensions, other variables come into play: the high surface-area-to-volume ratios of micro- and nanoplastics allows for the adsorption of contaminants and formation of biofilms, and additives – including plasticizers, flame retardants, antioxidants, and stabilizers – incorporated into commercial plastic may be released in organisms.

Despite the severe concern, the study of both their detection and collection, especially for nanoplastics, is still embryonal, and even their definition is not unanimous. The term microplastics is used for fragments with sizes between

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Enrico Rampazzo, born in Verona in 1973. He completed his PhD in chemistry at the University of Padua under the supervision of prof. Umberto Tonelatto and prof. Fabrizio Mancin. He was a postdoctoral fellow under the supervision of prof. Luca Prodi and prof. Marco Montalti, and then fix-term researcher at the Photochemical Nanosciences Laboratory of the University of Bologna (Italy). He is now associate professor of general and inorganic chemistry at the University of Bologna. His research focuses on the development of luminescent dyes, sensors and (electro)luminescent systems based on supramolecular systems, dye doped silica nanoparticles and polymers.

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1 μm and 5 mm, while the size definition of nanoplastics is still discussed and upper limits of 100 nm or 1 μm have both been proposed. This could appear only a semantic discussion but definitions, instead, have important consequences on guidelines and legislative proposals at local, national, and international level.

Many regulations in the form of international conventions and treaties are in force to prevent, mitigate and remove pollution. All other regulations are mostly devoted to plastic litter and other materials entering the environment and they mainly concern marine and fresh water. Unfortunately, these instruments suffer of two main problems: lack of coordination and of compliance. Even if regional needs must be considered for effective norms, ecosystems are all networking and interlocked and global cooperation is fundamental to successfully address pollution, therefore common laws fulfilling existing gaps are necessary.

An overview on EU regulations shows that (micro-)plastics are explicitly targeted only in a very small bunch of legislations, having as concerned environmental compartment marine water, fresh water and soil. Few legislations specifically refer to microplastic and they are often dedicated to regulating the presence of primary microplastics in consumer goods such as cosmetic products. In this framework, it must be noted that specific norms on MNPs (new or implementations) directly involved in the safeguard of human health are completely absent, and this is a consequence of the poor scientific knowledge on the impact that MNPs can have on human health, and in general on living organisms. As an example, in 2019 the World Health Organization (WHO) reviewed with a report the presence of microplastics in drinking-water pointing out, together with the raising of this problem, the uncertainties in terms of definitions, studies, sources, and impact on human health; as a conclusion, the WHO did not recommend frequent monitoring of microplastics in drinking water since no pieces of evidence were found to indicate a concern for human health. Similarly, the Science Advice for Policy by European Academies consortium (SAPEA), with its 2019 report reviewed the current evidence on health, environmental and societal impacts of MNPs pollution. This report, again, indicated the absence of evidence on the impact of microplastics on human health, but also pointed out the insufficient status of methods for measuring exposure and hazard, and the occurrence of scarce quantitative data – often of poor quality – that “does not allow to conclude that risk is either present or absent, with sufficient certainty”. The magnitude and the awareness of this problem inside the EU are however evident considering that just a couple of years later (2020), the control of microplastics in drinking water has become a priority for the European Union with the EU directive 2020/2184. This directive asks for the harmonization of the analytical methods of emerging pollutants – such as microplastics – by 12 January 2024, and for the adoption of a method to measure microplastics in water for human consumption, and ask the European Commission - no later than 12 January 2029 – to submit a report to the European Parliament and the Council on the potential threat to human health produced by microplastics and other emerging pollutants found in water for human consumption.

This makes clear the fundamental role of researchers in the next future, designing analytical procedures for the targeting of micro-plastics and hopefully, for nano-plastics. These approaches will need to be under quality control and to allow understanding the sources and presence of microplastics in drinking water, the effectiveness of water treatment processes, and the potential return of microplastics to the environment following the waste treatment procedures, including the reintegration of sludge to agricultural land.

2. Sample Collection and Pre-Treatment

The collection of representative samples is the first critical step in the identification and quantification of MNPs in the environment either in sea surface, or water columns or sediments. Sampling approaches can be divided in three categories: selective sampling, bulk sampling, or volume-reduced sampling. Selective sampling consists of direct extraction from the environment of items recognized by naked eye (larger microplastics 1–5 mm) usually on the surface of sediments but with a great risk of overlooking them when mixed with other debris. Bulk sampling collects the entire volume of the sample without reductions during the process: more precise but large volumes must be handled. In volume-reduced sampling the volume reduction of the bulk sample is obtained, for example, filtering with nets and only the portion of interest is preserved. The main advantage is for low concentration pollution when large volumes of water can be sampled quickly, but – depending on net mesh size and opening areas – sampling may not be very efficient, and smallest particles, in particular nanoplastics, are normally excluded from sampling.

After collection, microplastics can be further separated from the matrix, often by density floatation through salt addition to render plastics buoyant. Flotation can achieve nearly complete separation in the millimetre size range but is rarely used and inefficient for smallest plastic particles since the buoyant force is low and surface fouling can significantly change the particle density. Alternative separation approaches are filtration through size fractionation or sieving through size exclusion, with different mesh sizes. Also sequential cross-flow filtration followed by an asymmetric flow field-flow fractionation can allow to sort particles by size and concentrate the smallest particles.

Finally, chemical digestion is widely used for the purification of sampled material, particularly from biological material, and requires treatment with oxidants at extreme pH (e.g., H2O2, also in combination with Fe3+, strong acids and bases). Its applicability is however limited by possible plastic reaction or degradation, particularly at high temperatures (> 80°C). Enzymes (e.g., proteinase K, chitinase, cellulase, lipase, amylase) can also be applied for more specific digestion of tissue, either alone or in combination with chemical digestants.
3. State of the Art for MNPs Detection

The detection of MNPs is still in its infancy, as evidenced by the absence of recognized protocols – performed under quality control – for detection and quantification of MNPs. As a consequence, a direct, quantitative comparison of the results obtained with different methodologies is not straightforward. Indeed, it is often even difficult to compare the results obtained – with the same technique – by different laboratories. This has also heavy consequences on the development of the legislation in the field that is still very limited. The analytical problem of MNPs detection and quantification might make a great step forward by exploiting the synergy between different analytical techniques, each able to provide all the different information needed for MNPs recognition and quantification. It is beyond the scope of this contribution to review all the analytical techniques available for MNPs detection; we have tried however to summarize the pros and cons of the main available ones in Table 1, to give a perspective of their common features and complementarities.

It is important to underline that many of the different entries listed in Table 1 are related to microscopy techniques. In these cases, since in theory single particles could be observed, an important parameter to report is the smallest observable dimension that can be counted. This limit could also be advantageously used to select the best sampling methodology.

Among the different methods used for MNPs detection, photoluminescence spectroscopy already plays a role. Yet, we strongly believe that due to its high sensitivity and versatility – which can be implemented using simple and cheap instrumentation components – photoluminescence-based techniques will gain a leading role in the future. For this reason, we describe here the state of the art and perspectives of this specific sector, hoping that this will contribute to address the problem of MNPs detection in a more efficient and conclusive way.

4. Detection of MNPs Based on Their Autofluorescence

The possibility to monitor a target analyte taking profit from its own luminescence properties can present several advantages and, in particular, the possibility to perform a direct measurement without the need of labelling or staining makes the analytical procedure streamlined and can avoid artifacts. This approach has been recently reported – although by very few groups – also for the detection of microplastics. For example, Biver et al.[70] proposed the use of autofluorescence, together with UV and reflectance index detectors, to distinguish, after gel permeation chromatography (GPC), two microplastic types used as reference compounds, namely polyethylene (LDPE-oxo) and partially oxidized low-density polyethylene (LDPE-oxo). The photophysical properties of these two kinds of polymers had been previously studied by other groups. In the case of PS, it was observed in 1,2-dichloroethane a fluorescence band with a

Table 1. Major analytical techniques for the detection, identification, and quantification of microplastics.

| Analytical technique | Size range | PROS | CONS |
|----------------------|------------|------|------|
| Visual sorting (light microscopy and stereoscopic microscopy) | > 500 μm | Cheap, fast, simple and in situ. | High possibility of false positive/negative, no chemical information. |
| Optical microscopy | > 0.5 μm | Simple and common, information on morphology. | High possibility of false positive and of missing small and transparent plastic particles, no confirmation of polymer composition. |
| Thermogravimetry | μm–mm | High resolution, couplable with other instruments. | Destructive. No information about particle number, size, morphology, or aggregation. |
| Gas chromatography/mass spectrometry (GC/MS) | ng–μg | Fast, limited sample preparation, chemical identification of polymer and organic plastic additives (OPAs) by their pyrolysis products, and possibility of quantification. | Destructive, complex reproducibility, manually placing into the pyrolysis tube. |
| Differential scanning calorimetry (DSC) | μm–mm | Cheap and simple | Destructive, results affected by particle size, additives, impurities, and branching of polymer chains. |
| Fluorescence microscopy (NILE RED) | μm | Cheap, fast, size and morphology information. | Mostly non-specific, fluorescence background and false positive, in most cases the information of chemical composition is very scarce. |
| Fourier-transform infrared spectroscopy and microscopy (FTIR) | > 30–50 μm | Fast, non-destructive, minimal preparation of the sample, accurate chemical identification, possibility of imaging, of coupling with a microscopy and of automation. | Expensive instruments, difficulty in comparison, interference by water, no interpretable spectra of thick, opaque, and coloured microplastics in transmission mode, reflection errors in reflectance mode, contact in ATR mode. No automated analysis of particles. |
| RAMAN microscopy and spectroscopy | > 1–2 μm | Fast, non-destructive, minimal preparation of the sample, accurate chemical identification, possibility of imaging, of coupling with a microscopy and of automation. | Expensive instruments, difficulty in comparison, interference fluorescence background. |
| Electron microscopy (SEM, TEM) | nm–μm | High resolution, size (nanoplastics) and morphology, Elemental composition information if coupled with Energy dispersive X-ray spectrometry (EDS). | Sample preparation, expensive instrumentation and time consuming. No chemical information without EDS. |
| Scanning probe microscopy | nm–μm | High resolution, possibility of in liquid analysis with atomic force microscopy (AFM). | Long and laborious, measurements possible only for specific particles or sections of the sample. |
maximum at 335 nm attributed to the formation of excimers among pendant phenyl groups,[71] while a much less intense peak at 283 nm has been attributed to a monomer emission. Allen et al. reported for additive-free grade LLDPE and HDPE[72] in powder and in polymer films a fluorescence emission, that presents an excitation maximum at 230 nm and an emission maximum at 340 nm, attributed to the presence of low levels of cyclic unsaturated carbonyl compounds of the enone or enal type. Interestingly, Allen et al. also reported a phosphorescence spectrum with an excitation maximum at 250 nm and a structured emission band in the 400–500 nm region, attributed to the triplet state of the carbonyl groups.

Biver et al. in their article reported that the dichloromethane dispersion of the two materials showed upon excitation at 260 nm different fluorescence spectra: the emission band of PS presented a maximum at 335 nm, in agreement with previous results, while the emission band of LDPE-oxo presented two peaks at 358 and 375 nm and a shoulder at 420 nm, that results, while the emission band of LDPE-oxo presented two wavelengths longer than 290 nm. To follow the GPC runs with fluorescence, the authors decided to use two different couples of excitation/emission wavelengths, i.e., 260/280 nm and 370/420; in the first case they were able to selectively observe in the GPC trace PS, in the second case only LDPE-oxo. To our opinion, this approach presents some possible drawbacks. As far as the first couple (260/280) is concerned, from one hand, the excitation wavelength can be absorbed by many possible impurities (additives, pollutants, biological species) also giving autofluorescence, causing possible interferences; on the other hand, the chosen emission wavelength is too close to the excitation one and far away from the fluorescence maximum, so that the scattered light can become the most important contribution to the observed intensity. As far as the second couple is concerned, the results obtained are different to those previously obtained, and the possible effects of additives should be considered.

Similar fluorescence bands in the 400–550 nm region have been obtained by Langhals et al.[73] exciting at 365 nm chloroform dispersions of Luran® (a copolymer of styrene, and polycrylonitile) and Ultramid® (a polyamide with glass fibre). In the same article, the authors described the possible identification of each polymer based on their different excited state lifetimes. This kind of approach, although requiring a relatively complex instrumentation, represents a very interesting possibility for distinguishing analytes (or different environments in which they are present) having similar emission wavelength, highlighting the great versatility of photoluminescence spectroscopy. The group of Langhals in collaboration with the groups of Dietrich has extended this approach[74] also using the phasor analysis that allows to present data in an interactive 2D plot, increasing speed of analysis and avoiding fitting algorithms.[75] In these cases, the excitation has been performed, yielding similar lifetimes, both at 440 and 470 nm. These articles, however, did not report additional spectral information (such as excitation and emission spectra and quantum yields) that could be crucial to a complete understanding of the observed data, especially considering that these wavelengths are not efficiently absorbed by many plastics.

Overall, although we believe that the detection of autofluorescence could be of interest for the analysis of MNPs, our vision is that a more complete photochemical and photophysical investigation is needed before this approach could be extended, to understand the contribution to the luminescence of each single component (the plastic, possible additives, and possible adsorbed pollutants), and their possible changes caused by ageing, including the one induced by light.

5. Staining

The most widely exploited luminescence-based strategy for the detection of micro and nanoplastics involves the use of fluorophores able to stain plastic particles, allowing their recognition with fluorescence techniques, in particular fluorescence microscopy (Figure 2).[76] All staining agents, as described below, share some aspects as far as the signal transduction mechanism is concerned. A first common feature they have is a high affinity toward plastics, so that the partition of the staining agent between the solvent (preferably water, but other solvents have been used) and the particle surface is shifted toward the latter system, allowing a high signal to noise ratio. This equilibrium is based on supramolecular interactions,
with hydrophobic interactions playing an important role, and for this reason depends also on the plastic material. Even if only few examples have been reported so far, the staining agent can be tailored for maximizing the affinity for specific polymers. A second, common staining aspect, is the change of the physical properties of the probe when interacting with the plastic particle: typically, an increase of the luminescence intensity or a change in the emission wavelength, both affecting also excited state lifetime. Solvatochromism, deactivation of self-quenching processes, or activation of AIE mechanisms have been exploited; the larger is the change the higher is the signal to noise ratio that can be obtained. Since also these effects can depend on the plastic type, they are used to differentiate among the different polymers. Finally, aggregation of the staining agent can also occur, a phenomenon that could create artifacts, especially in case of luminescent aggregates, and that must be always carefully evaluated.

5.1. Nile Red

The workhorse in staining methods of micro and nanoplastics is by large the Nile Red (NR, i.e., 9-diethylamino-5H-benzo[α]phenoxazine-5-one, Scheme 1), a hydrophobic fluorophore that specifically binds to neutral lipids, allowing in situ staining, becoming strongly fluorescent only in the presence of a hydrophobic environment. NR presents an additional, very interesting characteristic, i.e., its solvatochromism: its fluorescence emission spectrum redshifts markedly as the polarity of the medium increases, which can be attributed to the twisted intramolecular charge transfer state of the dye molecule. This behaviour can be an advantage in plastic particles analysis, allowing the categorisation of plastics into types based on their general hydrophobicity, for example polyolefins, polyaromatics, polyesters and nylons, or providing a useful indicator to evaluate residence time in the environment because of changes in surface properties caused by oxidation or biofouling. However, the emitted colour of Nile Red can be affected by surface contamination from the environment, which may change the hydrophobicity of plastics.

Together with its favourable photophysical properties, NR presents also some drawbacks limiting its possible analytical performance. The first one is represented by the fact that NR tends to form aggregates due to intermolecular interactions at high concentrations, resulting in a decrease in fluorescence intensity or even in its total quenching. Therefore, the fluorescence intensity of a sample of microparticles first increases and then decreases with rising Nile Red concentration. In addition, higher dye concentrations increase the background signal due to unspecific adsorption on other materials or self-aggregation driven by the poor water solubility, resulting in unspecific noise. For this reason, it was experimentally proved by different authors that the optimum dye concentration is between 0.1–10 μg/mL ensuring a good balance among visibility, speed, and background signal.

Interestingly, with suitable staining protocols, the recovery rate of plastic particles has been, in some cases, proven to be around 95%–100%. After NR staining, fluorescence is sufficiently clear to enable effective identification of PE (polyethylene), PP (polypropylene), PS, expanded PS, PC (polycarbonate), PU (polyurethane), EVA (ethylene-vinyl acetate), and nylon-6, while PVC (polyvinyl chloride), PET (polyethylene terephthalate), PA (polyamide) and tire rubber present dim fluorescence or no fluorescence, although PET and PA could be observable thanks to their intrinsic blue fluorescence (Figure 3).

Moreover, poor fluorescence can be observed also in several textile fibres, such as viscose, nylon, and polyester, while cotton and wool present no fluorescence. Since the dye is adsorbed onto plastic surfaces, polymers with larger surface area, for example mesoporous structures or irregular shapes, tend to be stained more brightly with NR compared to more spherical particles.

Another possible drawback is represented by the fact that NR is not specific for plastics, being able to stain also other

![Scheme 1. Molecular structure of Nile red and of three derivates.](image)

![Figure 3. Comparison of fluorescence from Nile Red stained HDPE (a), PC (b), PU (c), PEVA (d), PVC (e) and PE (f) polyester and non-stained fibres of polyester (g), PET (h) and polyamide (i). Reproduced with permission from Ref. [59]. Copyright 2016, Elsevier.](image)
organic materials, and for this reason its use can lead to an overestimation of plastic content in environmental samples. A possible solution is a pre-treatment of the sample to remove biogenic matter with purification and separation stages. An example of this approach is described by Vermeiren et al., who developed a protocol consisted of three steps: first, chemical digestion of the organic matter with Fenton’s reagent, secondly a density separation with ZnCl₂ solution, using a novel column with a top overflow, and finally the NR staining of the plastic particles, which were analysed with epifluorescence microscopy and then with FTIR microscopy to reveal polymer identity. This procedure obtained recovery rates of microplastics above 90%, and μTIR confirmed that 91.7% of the stained particles were plastics.

Another possible approach to separate fluorescent plastic particles from organic matter in environmental samples is the co-staining with two different dyes: NR to stain plastic particles and a second fluorophore that can bind selectively to biological material. This is the approach followed by Stanton et al., who compared the efficacy of staining with NR alone to co-staining with NR and 4',6-diamidino-2-phenylindole (DAPI) in samples of drinking water and fresh water. After the staining, NR was observed in green fluorescence (excitation wavelength, 430–490 nm; emission wavelength, 510–560 nm), and DAPI was observed in blue fluorescence (excitation wavelength, 355–405 nm; emission wavelength, 420–480 nm) with a fluorescence microscope. Considering all fragments that fluoresced in blue wavelengths of light as biological matter since stained with DAPI, they estimated the number of false positives resulted from Nile Red staining. With this approximation, they found a high overestimation of microplastic abundance, ranging from 10.8% (in freshwater samples treated with H₂O₂) to 100% (in drinking water). However, the aim of this work was only to illustrate possible limitations of Nile Red staining method in environmental samples, not to provide a universal estimation of false positive rates, since the possibility of a blue autofluorescence or of interaction between DAPI and plastics have not been considered as significant factors. Besides, Nel et al. hypothesized that the overestimation could result from the lack of a threshold limit based on polymer specific particle pixel brightness, which varies between polymer type, shape, size, colour and by staining procedure, leading to the counting of all particles that showed fluorescence and subsequently to their overestimation.

The approach of co-staining to distinguish plastic particles from organic matter was investigated also by Michelaraki et al., who studied the combined use of NR and Methylene Blue on two oceanic soil samples, pre-treated with density separation and filtration. Compared with Nile Red alone, Methylene Blue reduced the fluorescence generated from biological matter, minimizing interferences, and reducing false positives.

A third possible limitation of all NR staining methods described above is that the dye molecules are just physically adsorbed on the surface of plastic particles, so they can easily be desorbed, leading to a decrease of fluorescence intensity. To avoid this, Lv et al. developed a staining method based on the thermal expansion and contraction of the plastic. These authors have based their approach on the fact that the dyes can enter inside the plastics at high temperature when the macromolecular chain network is loosened. If the sample rapidly returns to room temperature, the macromolecular chain assumes a denser structure and dye molecules remain encapsulated into the plastics. This method was tested on three different fluorophores: Safranine T, Fluorescein isothiocyanate (FITC) and NR on four types of plastic particles (PS, PE, PVC, and PET).

The best results were obtained with NR: using this dye the fluorescence intensity of all microplastics increased by increasing the staining temperature and time and the recovery rate was practically 100%, except for the sample incubated with the dye at room temperature. After two months, all stained microplastics were observed again, obtaining no significant difference in the fluorescence intensity, since dye molecules were encapsulated inside the plastics and they could not easily undergo desorption.

Sturm et al. also performed some modifications of NR molecular structure to achieve greater selectivity for plastic particles and more intense fluorescence. They tested the performance of NR staining compared with three derivatives (Scheme 1), obtained replacing the ethyl substituents present in Nile Red with propionic acid groups (NR1) to increase the affinity for polar polymers like PVC, with n-hexyl groups (NR2) or branched ethylhexyl groups (NR3) to increase the affinity for lipophilic microplastics. The authors tested various microplastics (polyethylene, polypropylene, copolyamide, copolyester, and polyvinylchloride) and natural particles (wood, chalk, and chitin). No one of the Nile Red derivatives led to a consistently positive effect, increasing the signal intensity for plastic particles and/or reducing the signal intensity for natural particles. To stain an environmental sample (sea salt, which is evaporated seawater) a combination of NR and NR3 in acidic water was chosen to differentiate between microplastics and natural particles. Since chitin showed a strong fluorescent signal, the sea salt samples were treated with hydrogen peroxide and chitinase to reduce the risk of false positives. Applying the combination of NR and NR3 on the environmental samples different particles were stained, but in some cases the fluorescence signal was of intermediate strength, preventing to distinguish natural matter from plastic. Since most of these ambiguities occurred at size ranges below 50 μm, the lower size limit was set to this dimension.

Fluorescence lifetime imaging microscopy (FLIM) can be also advantageously used to allow polymer identification, with the advantage that lifetimes are usually not affected by experimental conditions, like scattering or dye concentration. This approach was followed, for instance, by Sancataldo et al., who analysed microplastics of low-density polyethylene, poly-styrene, polyethylene terephthalate, and polyamide (nylon) with spectrally resolved confocal fluorescence microscopy and FLIM. FLIM data were analysed by the already mentioned phasor approach, allowing a simplified plotting of the fluorescence lifetime distribution, without any modelling or fitting procedure. In this way, since NR fluorescence showed peculiar behaviours based on the polymer matrix, the four...
plastic types were identified, also when different microplastics were simultaneously present in the same sample.

Another technique that can analyse stained plastics coupled with fluorescence microscopy is the single-particle tracking, which allows the identification of the number and the size of nanoparticles. For example, Molenaar et al.\textsuperscript{[86]} combined these two techniques to analyse commercially available PS beads with a nominal diameter of 400 nm, 200 nm, 100 nm and 40 nm and plastic particles obtained by grinding a commercially available disposable PS plastic cup into a powder with a commercial kitchen mixer. Studying these nano-plastics, they found that mixed ratios of differently sized particles can be recovered, and they determined the limit of detection in terms of size and concentration, i.e., 45 nm of hydrodynamic diameters and 2 x 10^6 particles per mL; lower concentrations can be investigated by including more frames in the analysis, for example refreshing the detection volume by introducing flow. They also analysed the particles that were present in the demineralized water left for 5 minutes at 95 °C in a plastic cup, limiting with a suitable filter the investigations to particles < 1 μm. Using NR staining coupled with single-particle tracking, they found a concentration of approximately 10^7 particles per mL, with size distribution peaks around a diameter of 160 nm and with a distinct tail toward larger sizes; Raman spectroscopy tests on concentrated samples showed analogous spectra compared to those obtained for the cup itself. Therefore, although this method does not provide information on the polymer type or on the size of the individual plastic particles, coupling NR staining and single-particle tracking can determine nanoplastic concentrations and size distributions. However, recovering both the particles size distribution and the particle number concentration is not trivial and may, depending on the sample, require separate optimization steps in data recording and analysis.

Microscopy techniques are not the only methods that allow a determination of the plastic identity of stained particles; for example, also macroscopic photoluminescence properties can provide an identification analysis if compared with proper reference polymers. Konde et al.\textsuperscript{[87]} for instance, studied NR staining of foils made of four polymer types (polypropylene and polyethylene as less polar plastics, polyethylene terephthalate and polyvinyl chloride as more polar plastics), after an optimized procedure the emission spectra of the four polymer types were collected and analysed, observing in particular the wavelength of the peak maximum showed in Figure 4: the spectral shift increased with the polarity of the polymer, allowing its identification.

Some studies were conducted to compare NR with other possible staining dyes. For example, Prata et al.\textsuperscript{[88]} tested eight fluorophores, i.e. Acidine Orange, Basic Blue 24, Crystal Violet, Lactophenol Blue, Neutral Red, Nile Red, Safranin-T, and Trypan Blue, with virgin polymers (LDPE, PP, PS, HDPE, PET, expanded PS, CA, PVC and nylon), weathered synthetic polymers (sediment samples, where polymers were identified by FTIR as HDPE, PE, PE, PP, EPS and CA), textile fibres (cotton, linen, polyester, cotton-polyester, polyamide, viscose, rayon and nylon), natural organic matter and even with filters of quartz, glass microfiber, nitrocellulose, mixed cellulose esters, black polycarbonate filters (PCTE) and C18 Octadecyl, to evaluate their possible interference in results. The authors concluded that among the eight staining dyes, NR presented the best results as it made most synthetic polymers and textile fibres fluorescent. Also, Maes et al.\textsuperscript{[89]} tested multiple dyes (Oil red EGN, Eosin B, Rose Bengal, Hostasol Yellow 3G and NR) for their ability to adsorb onto plastics and they again found that NR was the most effective in terms of adsorption and fluorescence intensity. It is not therefore surprising that NR is the most used fluorophore as staining dye for plastic particles analysis and several articles investigated its applicability, determining the optimal staining conditions. Together with the limitations described above, we think that also photobleaching can be considered an important issue when excitation is performed with high power light sources and that excitation and emission wavelength should be carefully selected starting from a complete photophysical characterization of the examined systems. In this context, we strongly suggest, to increase the reproducibility of data obtained from different laboratories, to use in all cases corrected spectra.\textsuperscript{[90]} Some attempts to consider all aspects affecting the analytical results using NR as staining dye, and thus to make results more reproducible have been reported,\textsuperscript{[91]} but further studied for this and other dyes are strongly needed.

5.2. Other staining dyes

Tong et al.\textsuperscript{[92]} tested the Rhodamine B staining method on five types of microplastic polymers, namely polyethylene, polypropylene, polystyrene, polyvinyl chloride, and polyurethane. Comparing the staining efficiency in different solvents, i.e., ethanol, distilled water, and acetone, these authors concluded that ethanol was the most appropriate solvent for staining the microplastics with Rhodamine B.

After assessing the fluorescence stability in various conditions, the compatibility of the staining method with Raman spectroscopy was confirmed collecting the Raman spectra of

![Figure 4. Normalized emission spectra and photos of PE, PP, PET and PVC, stained with 20 μg/mL of Nile Red in acetone and ethanol at 50 °C for 10 min., showing the different emission colour due to the solvatochromism of the probe. Reproduced with permission from Ref. [87], Copyright 2020, Elsevier.](image-url)
the five types of microplastics polymers using a micro-Raman spectrometer. Finally, the efficacy of the Rhodamine B based staining method was demonstrated also on an environmental sample (river water).

Rhodamine B, though, is a bright dye also in water, which leads to a strong fluorescent background. Prodi et al. have shown that hyaluronan functionalized with Rhodamine B (Scheme 2) forms nanogels in which the luminescence of rhodamine dyes is highly quenched. In a recent patent, these nanogels have been demonstrated to stain micro and nanoparticles with high affinity, leading to a local increase of concentration and quantum yield of Rhodamine B dyes, resulting in bright emission spots localized on the plastic surfaces standing out of a dark background. This enhanced contrast allows for the sensitive detection of nanoplastics down to the 100 nm size range.

Karakolis et al. tested textile dyes as candidate labels for microplastics. Between the several commercially available disperse dyes (iDye) three dyes with different excitation/emission features were selected: iDye pink (pink dye), iDye blue (blue dye), and Rit DyeMore Kentucky Sky (Kentucky dye). Thanks to the lack of overlap of the fluorescent spectra, the three dyes could be detected simultaneously. For the staining process, plastics – with different chemical compositions and shapes – were added to aqueous dye solutions and heated at 70°C for two hours in darkness then cooled down and filtered and rinsed with water. Relatively strong fluorescent signal was achieved with PS, PET, and PVC fragments, HDPE microspheres, PET, and PAN fibres but not with LDPE and PP. This poor staining for LDPE and PP was attributed to impurities or additives in the plastics that may affect dye staining.

One main advantage of the use of these commercial dyes was the good stability under different environmental conditions.

Another example of fluorescent probe for micro- and nanoplastic analysis is presented in the work of Nakamura et al., who prepared luminescent water-soluble networks containing coumarin as fluorophore by using polyhedral oligomeric silsesquioxane (POSS) as a cross-linking point (Scheme 3).

This structure, dispersed in water, showed bimodal light-emission bands, in the blue region and yellow region of the visible spectrum. In particular, the emission changed from yellow to blue upon the introduction of plastic particles of PS, PLA and PMMA. Interestingly, it was not observed any effect caused by the presence of, potentially interfering, silica particles. In addition, the intensity of the blue-light-emission band steeply increased with the decrease of plastic particles diameters, showing that this method is suitable not only for the detection of particles but also for size discrimination. Moreover, the responsiveness of this probe was greatest for PS, followed in order by PLA, PMMA and silica, a trend corresponding to the hydrophobicity at the particle surfaces. On the other hand, a weakness of this probe was the low detection limit that was several tens to hundreds of micrograms of plastic particles.

An additional example of fluorescent probe for micro- and nanoplastic analysis is the one proposed by Montalti and coworkers, who developed a fluorogenic probe based on perylene-bisimide (PDI) (Scheme 4) for the direct detection of microplastics in water, showing a ratiometric behaviour in case of PVC particles.

The fluorophore was functionalized with two hydrophilic diethylene glycolic chains terminated with amino groups that are protonated at neutral pH to increase the hydrophilicity of the molecule compared with PDI, which presents poor solubility in water.

This synthesis can be conducted in few minutes by a one-pot reaction using a conventional microwave oven, therefore the proposed fluorescent sensor is easily producible on large scale with a cheap and environmental-friendly process. The probe exhibited an intense green fluorescence in DCM that is strongly quenched in water at neutral pH due to the formation of aggregates presenting a red fluorescence characterized by extremely low fluorescence quantum yields and very short excited state lifetimes (< 0.1 ns). Interestingly, PE, PET, PP,
PS, PMMA or PTFE microplastics added to water solutions of the fluorogenic probe turned brightly green emissive, while PVC presented a peculiar red emission. Data showed that the fluorescence intensity and colours of stained PVC were concentration-dependent. Indeed, the total average intensity of the red component and the ratio \( I_R/I_G \) between this component and the emission observed in the green progressively increase with the probe concentration, until the optimal concentration of 8 \( \mu M \) is reached. This demonstrates that this fluorescent probe can be used as a ratiometric sensor for PVC micro-particles analysis, making the identification of the target analyte largely independent on the experimental conditions.

6. Conclusions and Perspectives

The huge problem of the immense amount of plastic that every year – at an increasing pace – is dispersed into the environment is coming to general attention in all its urgency, and derives from decision and behaviour taken by humans. For this reason, it is by its nature a very complex problem involving environmental and health aspects but also having a huge impact from the industrial and economical point of view. In particular, micro- and nano-plastics pose a great concern, because of their tiny dimensions, for their possible impact on flora and fauna, including mankind. Unfortunately, the ability to track MNPs is not advancing at the same pace and, as a result, although many analytical techniques have been proposed for this purpose, satisfactory analytical protocols have not yet been developed for microplastics and they are quite far to be obtained in case of nanoplastics. The lack of sampling and analytical protocols under quality control has also an influence on the possibility to control and regulate this field, shifting even further away in time the possibility to address the MNPs pollution. This environmental and analytical problem is huge, and will probably be solved in the future by exploiting the synergy between different and complementary analytical techniques.

In this context, we believe that luminescence-based techniques will provide an important contribution, since they offer very high sensitivity and versatility that, in some cases, can be obtained with relatively simple (and that, for this reason, can be used also by untrained personnel) and cheap instrumentation.

Interestingly, some kinds of plastics show distinct autofluorescence that can be used for their detection. However, their excitation and emission wavelengths are typically located in the UV range, and for this reason, the analysis can be tricky since inner filter effects and background fluorescence can dramatically affect the reproducibility and quality of the signal-to-noise ratio. In addition, possible additives and contaminants can give fluorescence signals in this region; a deeper photophysical investigation is, to our opinion, necessary to obtain more reliable data from this approach that cannot be, in any case, valid for all polymers.

Much better analytical performances have been obtained staining the target sample with suitable dyes, and in particular Nile Red, by far the workhorse of this kind of approach. It offers, in fact, interesting advantages: high brightness when in contact with MNPs, and photophysical properties, including excitation and emission wavelengths and excited-state lifetime, that – since they depend on the environment around the dye – can help in the identification of the chemical nature of the observed plastics. However, the possible performances are somehow limited, although many research efforts have been conveyed on this problem, by some issues that are not completely addressed yet, and in particular (i) the formation of aggregates that affect the luminescence properties and are a source of background noise, (ii) a relevant affinity also for other organic matter, that is unavoidably present in many analytical matrices, yielding possible false positives, while (iii) the affinity for plastics is sometimes not sufficient for stable staining. In addition, to our opinion a more detailed consideration of the photophysical stability of the system should be performed. Despite these issues, Nile Red remains the most used staining dye, although very interesting systems, namely a polyhedral oligomeric silsesquioxane and a fluorogenic probe based on perylene-bisimide, have shown a ratiometric behaviour for PS, PLA and PMMA, and PVC, respectively. In perspective, we believe that the next research steps should be devoted to the design and development of bio-compatible, water soluble probes showing a strong OFF-ON or ratiometric behaviour, endowed with a higher affinity towards plastics and low toward organic (that can be otherwise addressed with an oxidizing pre-treatment) or inorganic interferents. In this sense, the results obtained with nanostructured probes – such as the above mentioned silsesquioxane, the perylene-based nanoaggregates and the fluorogenic hyaluronan nanogels – lay the ground for a promising strategy to increase the overall analytical performance, since they have shown to be a step further in all these required properties.

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