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SHORT RESEARCH AND DISCUSSION ARTICLE

# Method of manufacturing and staining microplastics for using in the biological experiments

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## Abstract

Nowadays, plastic pollution attracts much attention both from society and scientists. The plastic pollution impact on the environment and human health requires assessment urgently, especially through experimental studies. However, such studies are still scarce because of the lack of standard methods for assessing their effects on living organisms. We have developed a process for manufacturing and staining PVC microparticles for using them in biological and ecological experiments. The electrospinning method has been used to manufacture PVC particles; their morphology and size have been analyzed. The obtained PVC particles are of narrow size range averaging 2–4 μm in diameter. They are successfully stained with the fluorescent dye Rhodamine B, which stands for the experiments performed in the seawater.

**Keywords** Polyvinyl chloride · Electrospinning · Microplastic · Staining · Rhodamine B · Copepods · Planktonic crustaceans

## Introduction

Microplastics are small plastic particles of a size range from 1 to 5 mm (Frias and Nash 2019). They are non-biodegradable and are derived from macroplastics such as polyethylene, polypropylene, polyvinyl chloride, polyester, and others (Zhang et al. 2019). Based on their origin, microplastics are classified into primary and secondary (Jaikumar et al. 2019). Primary microplastics are intentionally manufactured for cosmetics, soaps, toothpaste, body washes, scrubs, etc. Secondary microplastics are formed due to plastic degradation under mechanical abrasion, UV irradiation, and weather aging (El Hadri et al. 2020). All kins of microplastics accumulate finally in the soils, surface, and oceanic waters; they are brought to the seabed as well, where they form significant deposits, affecting negatively marine ecosystems (Curren et al. 2021). Microplastics may greatly affect living fauna and human health, although few in vitro studies have been performed, differing much in the experimental approach, making the data comparison quite tough (Dick and Juliette 2021).

In nature, deposited and suspended microplastics are mixed with various organic and inorganic compounds and particles of natural origin; hence, the process of isolating microplastics is quite challenging (Lusher et al. 2020). In addition, a mixture of different kinds of polymers of a wide size range makes it impossible to assess reliably any effect of a certain type and size group of microplastics on the living organism experimentally. Procedures for manufacturing microplastics of less than 10 μm for biological experiments are still under development. Electrospinning (electrospayring) is an effective method to manufacture microplastics, allowing particles within a narrow size group (Jaworek 2007). The advantage of electrospinning is that it is possible to create extremely small particle, which can even be reduced to nanometer size, by adjusting the technical parameters of the electrospinning process such as voltage and flow rate (Jaworek and Sobczyk 2008). In addition, this method prevents the coagulation of polymer droplets, due to the repulsion of the charged droplets.

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Plastic particles often have an indistinct color, which makes it a difficult task to detect them in the body. Such standard methods for determining the presence of plastic particles in organisms as Raman spectroscopy and GC-M pyrolysis require much time and are quite expensive (Sturm et al. 2021). Instead, staining the particles with a certain dye is one of the effective and low-cost methods for tracking their accumulation in the tissues and organs. The dye must meet the requirements of high selectivity and good fluorescence. Nile red is the most commonly used dye for staining microplastic (Shim et al. 2016; Erni-Cassola et al. 2017; Sturm et al. 2021). Recently, fluorescent dyes of different colors have been used as well (Karakolis et al. 2019). Rhodamine B is a fluorescent dye characterized by high fluorescence emission and good fluorescence stability in different environments, such as water, acid, and alkaline (Tong et al. 2021). It has been successfully used already to stain polystyrene in order to assess the toxicity of plastic particles on living organisms (Qu et al. 2018). It should also be noted that small amounts of Rhodamine B are not harmful to biological objects.

We have manufactured polyvinyl chloride (PVC) particles of small size (less than 10 μm) by the electrospinning method and stained them with Rhodamine B. These particles have been tested in the experiments with living planktonic crustaceans to assess dye stability and particle attractiveness for this kind of living test objects.

## Materials and methods

### Polymer and chemicals

Polyvinyl chloride (PVC) with Fickenscher's constant 58 was obtained from Shintech Inc. (Houston, TX, USA). Dimethylacetamide (DMA) (puriss.) and isopropyl alcohol (p.a.) were purchased from JSC "ECOS-1" (Moscow, Russia). Rhodamine B (p.a.) was purchased from SC "LenReaktiv" (Saint Peterburg, Russia).

### Manufacturing of PVC particles

PVC particles were manufactured by electrospinning using NANON-01A (MECC CO., LTD., Japan). PVC solutions 5 wt. % were prepared for fabrication particles by completely dissolving 0.5 g PVC in 10 mL of DMA. The electrospinning conditions were as follows: applied voltage of 20 kV, the solution feed rate of 0.5 mL/h, the distance between needle and collector of 15 cm. A Petri dish of 15 cm diameter filled with isopropyl alcohol was placed on a flat collector to collect the resulting particles. In order to study particle morphology, a blank slide (Levenhuk G50) was placed on the collector surface, and electrospinning was carried out for 3 min. The suspension containing PVC particles and isopropyl alcohol was transferred to a 50 mL vial for further experiments.

### Staining particles with Rhodamine B and obtaining dry PVC particles

Final 10 mg/L Rhodamine B solution was obtained by dissolving 0.05 g of Rhodamine B in 5 mL of isopropyl alcohol; similar concentrations have also been used for other dyes (Maes et al. 2017; Lv et al. 2019). This solution was added into a vial containing PVC particles. In order to enhance the diffusion of dye into the PVC particles, the mixture was blended at 70 °C for 2 h using a magnetic stirrer. Then, the mixture was under ultrasonic cavitation for 1 h to enhance the dye adhesion on the particle surface.

After the PVC particles were stained, the excess dye was washed out. First, the particles were let to settle down, and the supernatant was removed with a syringe. After removing most of the supernatant, 10 mL of isopropyl alcohol was added to the suspension, which was again shaken by ultrasonic cavitation to avoid polymer agglomeration. The particles were then allowed to settle, and the supernatant was removed as described above. The process marked by the asterisks was repeated several times until the supernatant was completely colorless. The isopropyl alcohol was then evaporated to obtain a dry PVC particles residue. The stained particles were transferred to a dark vial for further tests.

### Characteristics of manufactured particles

The dynamic viscosity of polymer suspensions was measured using a rheometer MCR 502 (Anton Paar, Austria) in a measuring cylinder. The measurements were carried out at 25 °C, at the shear range from 0.1 to 100 $s^{-1}$.

Measurement of the electrical conductivity of the suspension was carried out by a WTW inoLab Cond 720 conductometer (Germany) with a WTW TetraCon 325 sensor. The measurement error did not exceed 0.5%.

The size distribution of particles was evaluated using a particle diameter analyzer ANALYSETTE 22 MicroTec plus Fritsch (Idar-Oberstein, Germany) with a detection range of from 0.08 to 2000 μm.

The morphology of particles was analyzed under an STM6 light microscope (Olympus, Japan) with an additionally mounted UV source.

### Testing PVC particles in the experiment with live animals

The experiment was performed in March 2020 at the White Sea Biological Station of the Zoological Institute of the

Russian Academy of Sciences. Live planktonic animals were sampled from the ice with Juday plankton net (opening diameter 37 cm, mesh size 100 µm). The net was towed from 25 m depth up to the surface. Simultaneously, 5 L native seawater was taken at a 5 m depth and then filtered in the laboratory through a 0.2 µm Nucleopore filter to remove most suspended matter and plankton organisms. Ten individuals of *Pseudocalanus* spp. (copepodite stage V) were added to the 100 mL vial with 0.1 g of dyed PVC particles; the experiment was set in ten replicates. The experiment lasted for 120 h at 2 °C (ambient water temperature in the sea). The particle sedimentation was prevented by gentle turning of the closed vials every 3–4 h. All the animals were alive at the end of the experiment. All the copepods were placed into 2 mL Eppendorf cups (separately from each vial), immediately fixed with 1% formaldehyde solution, and stored at 4 °C prior to fluorescent analysis.

## Results and discussions

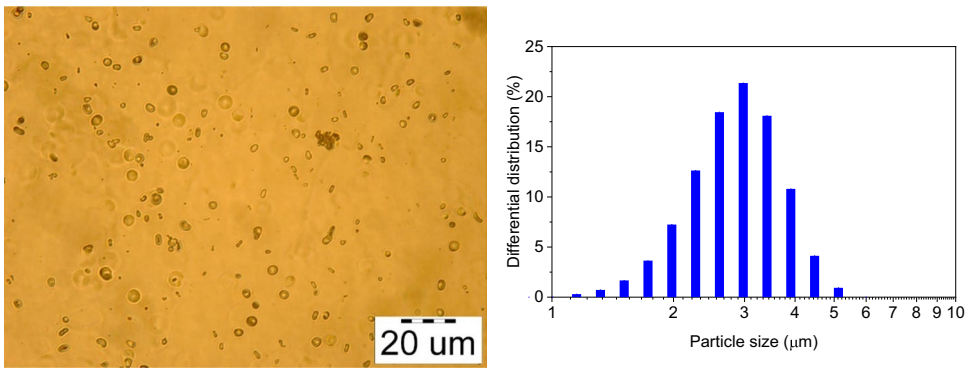
The electrospinning process is usually used to manufacture nanofibers. However, electrospinning is a process that generates both particles and fibers simultaneously. According to our previous study, the PVC solution concentration determines that fibers, particles, or both will be produced (Pham et al. 2019). When the concentration of PVC solution is less than 10%, this results as producing plastic particles of a rather tiny size. The properties of the solution (viscosity and electrical conductivity) greatly influence the electrospinning process as well. Viscosity depends on the molecular mass and the concentration of the polymer in the solvent. Both viscosity and conductivity of PVC solutions in different solvents are very low. The viscosity of the 5 wt.% PVC solution in DMA is 8.21 mPa·s. The electrical conductivity of 5 wt.% PVC solution in DMA is 0.9 µS/cm. Low viscosity promotes forming particles instead of fibers during electrospinning.

During electrospinning, the particles are manufactured on the collector surface; they are bonded together to form a film. A Petri dish containing isopropyl alcohol has been placed on the collector surface to collect the particles and separate them simultaneously to avoid aggregation. PVC is a hydrophobic polymer, so when the polymer droplets come into contact with isopropyl alcohol, they harden immediately and prevent their self-adhesion.

The morphology and size range of PVC particles obtained by the electrospinning process was quite homogenous (Fig. 1). A narrow size range characterized these PVC particles, and it is in the range averaging of 2–4 µm. This size range allows to use them as model microplastics in biological experiments since this size group is the most abundant in the marine environment due to their linear dimensions (Zobkov and Esiukova 2018).

PVC particles are characterized by pronounced luminescence after staining with Rhodamine B dyeing (Fig. 2a). However, this staining method has several limitations for applying certain counting methods, particularly fluorometry, since Rhodamine B has its own fluorescence; hence, using a laser analyzer sensor will be ineffective. In order to check if the PVC particles successfully keep their size after the staining, we have used light microscopy with a UV source. According to these observations, the size of most freshly manufactured and stained particles ranged from 4 to 6 µm (Fig. 2a). However, PVC particles also tended to aggregate (Fig. 2b).

We have also tested if the properties of the PVC particle suspension changed over time. After 2 weeks of natural sedimentation of suspension, the PVC particles agglomerated and formed large particle clusters, reaching 100 µm in diameter (Fig. 3). This process may negatively affect the effectiveness of biological testing, although such agglomeration is not stable. Therefore, we recommend either using freshly prepared suspension from the dried material (as described above in the section "Staining particles with Rhodamine B and obtaining dry PVC particles") or destroying polymer aggregates in the suspension using ultrasonic cavitation shortly before the experiment starts.




(a) (b)

**Fig. 1** Morphology and size distribution of freshly manufactured PVC particles

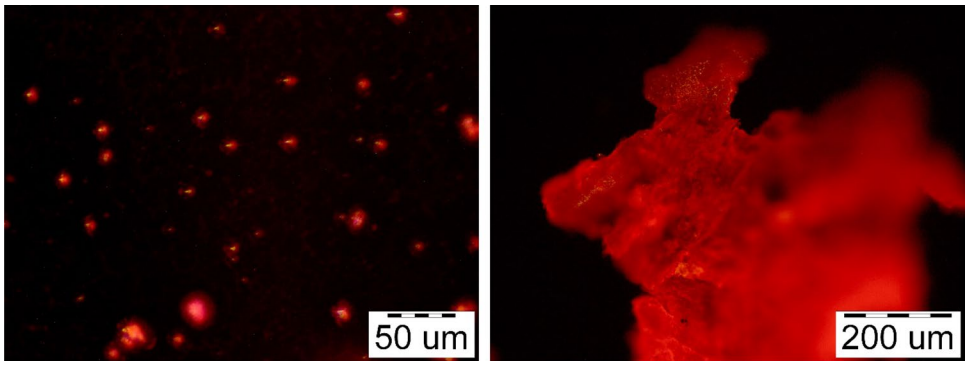



**Fig. 2** PVC particles fluorescence after Rhodamine B staining: **a** freshly prepared suspension; **b** large aggregation of particles after two weeks of natural sedimentation

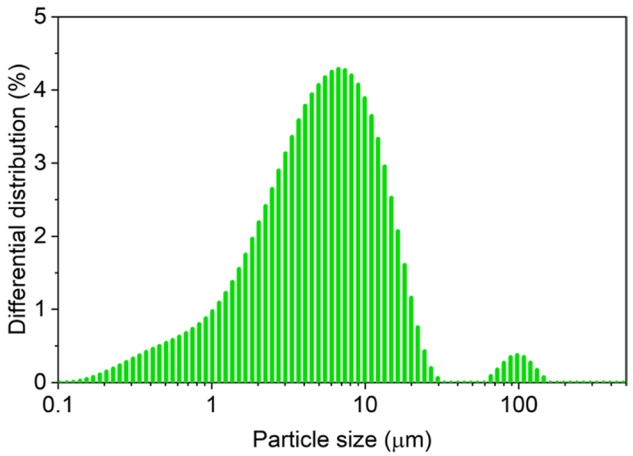



**Fig. 3** Size range of PVC particles after 2 weeks of natural sedimentation of a suspension

The feeding behavior of marine copepods of genus *Pseudocalanus* was analyzed using PVC stained particles. Copepods are the most numerous and diverse group inhabiting marine, brackish, and freshwater environments; they are widely used as model objects, and a large data on their feeding peculiarities and standard methods to study that makes it possible to assess the effect of artificial food items reliably (Harris et al. 2000). Copepods are known to ingest microplastic particles actively (Cole et al. 2013), which totally fit the goal of our study. Small copepods are characterized by a relatively transparent body, which allows tracking the fluorescent markers even inside the animal without any additional procedures (dissecting). The gut is straight and has no bends, so this helps to obtain quantitative feeding characteristics with minimal errors due to the absence of overlapping fluorescent particles.

The average size of *Pseudocalanus* spp (copepodite stage V) in the White Sea is about 450 μm (original measurements, no raw data is provided). The copepods of this size prefer the phytoplankton and microzooplankton of size range from 3 to 12 μm (Poulet 1974). The egestion rate (i.e., the time of retaining the food in the gut) in this species is less than 24 h, sometimes reaching just a few hours (Bedo et al. 1990). Therefore, after 120 h of exposure, only the PVC particles might be found in the guts if the animals feed on them. The copepods in our experiments were actively feeding on the suggested food substitute (Fig. 4) and were alive after a quite long experiment duration, which allowed us to consider this type of manufactured and stained microplastics as a good model object in the biological experiments.

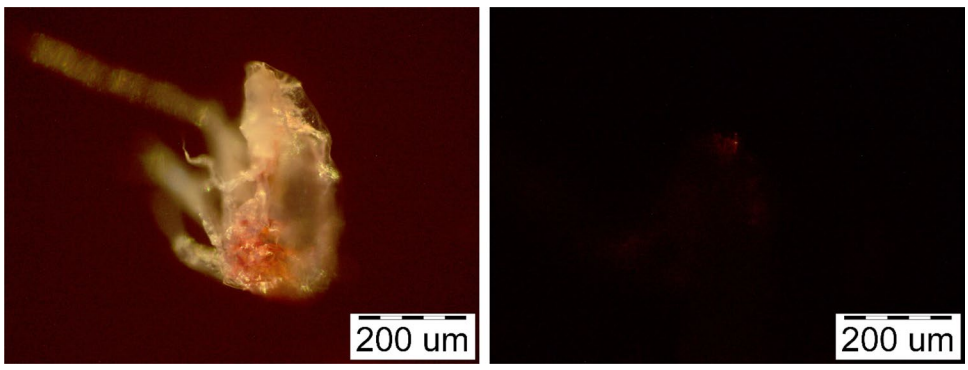



**Fig. 4** Cross section of *Pseudocalanus* spp. (gut area) with digested dyed PVC particles (**a**) and the body of *Pseudocalanus* with a red glow area, under UV (**b**)

## Conclusions

The PVC particles with an average size in a range of 2–4 μm were manufactured by the electrospinning method using the PVC solution of low concentration (less than 10%). Staining with Rhodamine B under ultrasonic cavitation increased the staining efficiency. UV light microscopy seems to be the best method for the qualitative and quantitative assessment of PVC particles. Light microscopy is low-cost and widely used worldwide. Testing the attractiveness of the manufactured particles in the experiments with live marine planktonic copepods evidenced good results. A complete procedure for PVC microplastics manufacturing, dyeing, and storing has been developed. The results of this study can be used to develop processes for each type of microplastics derived from different polymers. On the basis of assessing the influence of microplastics on the basis of different polymers on microorganisms, it is possible to predict the influence of natural microplastic mixtures on microorganisms.

**Author contribution** Mayya V. Uspenskaya and Roman O. Olekhnovich conceived of the presented idea. Pham Le Quoc, Maria I. Fokina, and Daria M. Martynova designed the study. Material preparation, data collection, and analysis were performed by Pham Le Quoc, Maria I. Fokina, and Daria M. Martynova. The first draft of the manuscript was written by Pham Le Quoc, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability** Not applicable.

## Declarations

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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