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Water-dispersible PVA-based dry microballoons with potential for biomedical applications

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ABSTRACT

This paper reports on the preparation and characterization of stable poly(vinyl alcohol) (PVA)-based dry hollow microparticles, readily convertible to gas-filled microballoons (MBs) in water suspension. The rehydrated MBs can be used as ultrasound contrast agents and for targeted drug delivery, while the dry MBs are suitable for encapsulation of biologically active gases. The MBs powder material is obtained by freeze-drying the as-prepared telechelic PVA-shelled MBs aqueous dispersion. The microstructure of the lyophilized MBs as well as of the starting and the reconstituted MBs in water suspension was examined using transmission electron microscopy (TEM), scanning electron microscopy (SEM), scanning transmission X-ray microspectroscopy (STXM) and confocal laser scanning microscopy (CLSM). STXM observations below and above the oxygen *K*-edge reveal that 80% of the MBs originating from the lyophilized particles are gas-filled. Moreover, local carbon *K* near-edge X-ray absorption fine structure spectroscopy (NEXAFS) measurements evidenced that the chemical composition of the polymeric shell is preserved during the freeze-drying process and subsequent shelf storage for at least more than one year.

1. Introduction

Over the past decade considerable research has been conducted to develop micrometer-sized bubbles (0.5–10 μm) as ultrasound contrast agents in medical investigation and clinical practice [1–5]. This is because the injected suspensions of gas-encapsulated microbubbles or microballoons into the bloodstream of a living body strongly reinforce echographic imaging, thus aiding in the visualization of vessels and internal tissue. Microballoons (MBs) based on air-filled denaturated albumin microcapsules, phospholipids, and liposomes containing gaseous SF_6 or perfluorocarbons are currently commercially available [5]. In recent years, polymeric microballoon MBs have been introduced in diagnostic imaging [6,7]. Furthermore, the injectable microballoons have also been explored as drug/gene carriers for controlled delivery [6,8–12]. This approach is associated with the transient cell membrane permeabilization upon ultrasound exposure (sonoporation) and release of encapsulated pharmaceutical agents at a specific location. Biologically relevant molecules can also be incorporated into the membrane that stabilizes MBs; charged drugs can be stabilized in or onto the surfaces of balloons by virtue of electrostatic interactions [10]. The fusion of *in vivo* diagnostics and therapeutic applications at the cellular level (also called *theranostics*) brings a demand for development of novel gas-encapsulated polymeric microcontainers with optimized properties. Among the most important characteristics of the efficient theranostic MB materials are the controlled size and shell thickness distribution, persistence in circulation, gas core protection, long-term storage stability and elasticity for controlling ultrasound damping behavior.

Poly(vinyl alcohol) (PVA) is a polymer of great interest for various pharmaceutical and biomedical applications due to its inherent non-toxicity, non-carcinogenity, good biocompatibility and physical properties such as compliance, elasticity and resistance to mechanical stress. At present, PVA hydrogels are effectively utilized for fabrication of contact lenses, wound dressing, coatings for sutures and catheters [13,14], and for use as synthetic articular cartilage [15]. Very recently, small PVA tubes were successfully used to replace a segment of an infrarenal aorta in rats [16]. In our previous works, we reported the preparation and characterization of air-filled MBs originating from a cross-linking reaction at the air/water interface of telechelic PVA

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solutions, i.e. PVA bearing aldehydes at the chain ends [17,18]. These balloons have an average diameter of 4.6 ± 0.4 μm and have shown a remarkable shelf-life of several months [19]. A study on the ultrasound properties of PVA-based MBs has been carried out recently [20]. The value of the pressure threshold, P_{thr}, at which the bubble shells cracked upon insonification has been found within the safety range of the mechanical index, MI, recommended for biomedical applications. Furthermore, these balloons have successfully been decorated with molecules like oligopeptides, amino acids, oligosaccharides, and polysaccharides on the external surface [21], enabling attachment of ligands to the surface of the MBs [22]. Biocompatibility of the MBs was examined recently by studying MTT essay on NIH 3 T3 mouse fibroblasts in the presence of PVA-shelled MBs. Cytotoxicity and proliferation tests of cells incubated in the presence of different amounts of MBs showed a very low impact on the cells viability [23]. Furthermore, it was demonstrated that PVA-shelled MBs can be loaded with nitric oxide, NO, and in *vitro* release of the gas was assessed revealing its efficacy as anticlotting agent [24]. A study on MBs loading and delivery of anticancer drug, doxorubicin, is in progress. Thus, PVA-based microballoons can be regarded as a very promising material for theranostics purposes.

In this study, we describe the fabrication and analysis of stable PVA-based dry MBs by lyophilizing an aqueous dispersion of air-filled MBs, which can be reconstituted in water to generate a microballoon-containing contrast agent. The dry state has recently been found as a suitable condition to encapsulate biologically active gases (e.g. nitrogen oxide, NO) in the microparticle core prior to dispersion in the carrier liquid [24]. This contribution also aims to investigate the freeze-dried state of PVA-based MBs and to monitor the potential changes in the morphology evaluating to what extent the physico-chemical properties of this system where maintained after the freeze-drying and resuspension processes. For that, microstructural visualization and *in situ* chemical analysis have been performed using a variety of microscopy and microspectroscopy techniques including transmission electron microscopy (TEM), scanning electron microscopy (SEM), scanning transmission X-ray microspectroscopy (STXM) and confocal laser scanning microscopy (CLSM).

2. Experimental

The synthesis of telechelic PVA-coated MBs was previously described in the literature [17,18]. In summary, a 2% PVA (Sigma, Germany) aqueous solution is added with $NaIO_4$ (RPE product, Carlo Erba, Italy) in an equimolar ratio with the head-to-head sequences of the PVA chains (about 1.5 mol%). The acetalization reaction was carried out at room temperature and pH 5. High-shear stirring is applied to the reaction mixture for 3 h with an Ultra Turrax T-25 homogeneizer at 8000 rpm. The cross-linking reaction is stopped by neutralizing the mixture, and the resulting suspension is dialyzed against Milli-Q water. Floating MBs were separated from the precipitated material and stored in a water-containing jar. For the preparation of freeze-dried MBs, aqueous suspensions of 10 mL MBs at a concentration of $10\ g\ L^{-1}$ were quenched at −70 °C and lyophilized at a reduced pressure of 30−40 mm Hg for three days.

Freeze-fracture TEM analysis was carried out by NanoAnalytical Laboratory (San Francisco, USA) using a JEOL 100 CX (JEOL, Japan) electron microscope. A MBs suspension was quenched using the sandwich technique and liquid nitrogen-cooled propane. Using this technique, a cooling rate of 10 000 K/s is reached, avoiding ice crystal formation. The fracturing process was carried out in a JEOL JED-9000 freeze-etching equipment (JEOL, Japan) and the exposed fracture planes were shadowed with Pt for 30 s and with C for 35 s (2 kV/60–70 mA, 10^{-5} Torr). Prior to examination the replicas were cleaned with concentrated, fuming HNO_3 for 24 h, followed by repeating agitation with fresh chloroform/ methanol (1:1 by vol.) at least 5 times.

The particle size and surface morphology of the dry MBs were examined using a Zeiss Supra VP55 high-resolution field emission microscope. The powder was deposited on a conductive double-sided carbon adhesive tape. The instrument was operated at 1 kV.

STXM studies on a dilute aqueous dispersion of the MBs were conducted at the PolLux beamline at the Swiss Light Source (SLS), Paul Scherrer Institut, Switzerland [25]. SLS storage ring runs at 2.4 GeV and "top-up" operation mode which guarantees a constant electron beam current of 400 ± 1.5 mA. The PolLux-STXM operates in the photon energy range between 200 and 1400 eV providing a spatial resolution better than 40 nm and an energy resolution of $\Delta E/E \approx 3 \times 10^{-4}$. Samples were imaged in transmission mode using a photomultiplier tube with a phosphor scintillator (Hamamatsu 647P). For *in situ* imaging the microballoons, we used the so-called "wet cells" where approximately 1 μL of uniformly distributed MBs aqueous suspension was sandwiched between two 100 nm thick Si_3N_4 membranes (Silson Ltd, UK), which were then sealed with silicone high-vacuum grease to maintain the water environment during the experiment. Carbon *K*-edge NEXAFS spectra were acquired in line mode, i.e., the transmitted signal was recorded while a line trajectory is scanned across the center of a MB at each value of the photon energy through the spectrum. The average spectra presented were calculated from 20 different single spectra taken from different MBs in the wet cells or from different dry MBs deposited onto Si_3N_4 membranes. The data processing was carried out using the aXis2000 software [26].

For CLSM measurements fluorescent dye RBITC (rhodamine B isothiocyanate, Fluka, Germany) was added to the MBs suspension at a typical concentration of 10 μM. The mixture was then stirred for 2 h. Floating particles were washed by resuspending in Milli-Q water several times. Confocal micrographs were collected with an inverted microscope, Nikon PCM 2000 (Nikon Instruments) with a 60×, 1.4 oil immersion objective. The 488 nm line of a 100 mW argon ion laser was used for fluorescence excitation. Images with a pixel size of 0.414 μm and an entire field of 512×512 pixels were recorded. MBs average diameter and standard deviation were determined over a set of 200 microparticles images, using ImageJ software package.

The freeze-dried particles subjected to SEM, STXM and CLSM analysis were stored for more than one year as a powder material in a fridge at 8 °C without any special concerns about the humidity. For STXM and CLSM analysis dry MBs powder was added to Milli-Q water (typical particle concentration ~10^{-5} wt.%) and the suspensions were gently shaken at 26 °C. MBs reconstitution was completed in 0.5 h and sample integrity was checked by observing microballoons floatation on top of the solvent. Immediately afterwards a droplet of suspension was taken for the STXM investigation. The overall time between rehydration and data collection (including preparation of the wet cell, backfilling the microscope chamber with He and focussing of the X-rays) was estimated to less than 30 min.

3. Results and discussion

The initial PVA-based MBs were prepared at room temperature at pH 5, hereafter named MB5RT, according to our synthetic protocol. For preparation of dry MBs, an aqueous suspension of MB5RT was frozen in liquid nitrogen and lyophilised. The microstructure of MB5RT in a frozen state was examined using freeze-fracture replica electron microscopy. Typical TEM images of the freeze-fractured MBs are shown in Fig. 1. As one can see, the images reveal the hollow core of the microballoons which is surrounded by the PVA fibrils protruding radially toward the outside. These microfibrils are the result of a cross-linking reaction at the water/air interface between the aldehyde chain terminals of oxidized PVA and the hydroxyl groups of the polymer backbone. It is believed, that the colloidal stability of the PVA-based MBs is due to the "hairy" structure of the supporting shell consisting of well-resolved polymer chains [17,18]. Thus, one of