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Ligands Tethering to Biocompatible Ultrasound Active Polymeric Microbubbles Surface

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Summary: Ultrasound imaging, also known as echography, is a widespread diagnostic tool in medicine. Handiness and versatility are established assets of this non-invasive imaging technique. Echogenic devices enhance the sensitivity of this method and formulations of novel systems is a growing field in biomedical research. In this contribution we describe the synthesis, the mesoscopic characterization, and the functionalization of microbubbles based on *telechelic* poly (vinyl alcohol) for a next-generation ultrasound imaging multifunctional device suitable for diagnosis as well as for therapeutic purposes.

Keywords: biomaterials; colloids; conjugated polymers; microbubbles; poly(vinyl alcohol)

Introduction

Gas filled hollow microparticles, i.e. microbubbles, are soft matter devices used in a number of diverse applications ranging from protein separation [1] and purification in food science,[2] to ultrasound imaging in biomedical field.[3] As far as this latter application is concerned, the number of ultrasonic imaging systems in operation outweighs that of systems dedicated to computer-assisted tomography by a factor of 10 (75,000 versus 7,000 units). The difference with respect to the number of magnetic resonance imaging spectrometers in operation (5000 units) is even larger. These figures [3] give an idea of the relevance of the research activity addressed in developing ultrasound contrast enhancers with improved diagnostic and therapeutic performances. A remarkable property of gas microbubbles is their efficiency as reflectors in ultrasound imaging, a nowadays established diagnostic method. In this respect, microbubbles are the main devices used for echographic investigations and are obtained with different kinds of materials and procedures: air-filled denatured human albumin, gaseous SF_6/phospholipids and perfluoropropane/liposomes microbubbles.[4] These ultrasound enhancers are commercially available. Despite the interest raised by such systems, the characterization of gas microbubble structures is still lacking and factors controlling their morphology and stability are not completely cleared out. In view of a ''next-generation'' ultrasound active device, an important issue related to a multifunctional use of the microbubbles is the possibility to use such devices as drug delivery systems. In this respect a significant asset of microbubbles is their injectability compared to oral administration performed with tablets or hydrogels.

Our aim is the formulation of devices behaving as ultrasound scattering enhancers using as starting material poly (vinyl alcohol), PVA, a synthetic polymer with good biocompatibility properties,[5,6] that matches some of the requirements imposed by a multifunctional use of the device. We have obtained aqueous suspensions of polymeric microbubbles by using a method that differs from those described in the literature. In our case, air is entrapped in a

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polymer shell obtained by chemically crosslinking, at high shear rate, modified PVA. Interfacial polymer networking was carried out by an acetalization reaction of aldehydic end groups introduced on PVA telechelic chains. The microballoon suspension, separated by flotation and stocked at room temperature in water, showed a shelf life of many months. In our opinion these type of microbubbles could be considered as a potential candidate for ultrasound investigations.

Considering the interaction of microbubbles with ultrasounds,[7,8] the formulation of a new class of mesoscopic systems functioning simultaneously as echocontrast and drug delivery agents can be envisaged and the echogenic properties, as the pressure threshold at which these microbubbles undergo inertial cavitation, are under investigation. In this context, loading and release of high and low molecular weight drug molecules can be envisaged, tuning the surface chemical properties by suitable functionalization methods. Therefore we have modified with different molecules the microbubbles surface as a preliminary step for a potential use of these systems in efficient tissue targeting and controlled drug release function. Moreover an alternative approach for the production of hollow microcapsules has been devised by exploiting the ability of PVA based microbubbles to modulate their wall permeability by changing solvent. Hollow microcapsules may also have applications in biology and medicine as microcontainers and microreactors for drug enzymes, DNA and other bioactive molecules.[9]

Experimental Section

Materials

Poly(vinyl alcohol) was a Sigma product. Number average molecular weight determined by membrane osmometry was $30{,}000 \pm 5{,}000$ g/mol, a weight average molecular weight of $70{,}000 \pm 10{,}000$ g/mol was determined by static light scattering.

Chitosan with number average molecular weight of $50{,}000 \pm 5{,}000$ g/mol and degree of acetylation of 0.1, β-cyclodextrin, amantadine, and poly (L-lysine) HBr, PLL, with a molecular weight of 7,500 g/mol were purchased from Sigma and used without further purification. Fluorescein isothiocyanate isomer I, FITC, Rhodamine B isothiocyanate, RBITC, 5-(4,6-dichlorotriazinyl)aminofluorescein, DTAF, were Fluka products. All inorganic chemicals and solvents were reagent grade products from Carlo Erba. Water was Milli-Q purity grade ($18.2\ M\Omega \cdot cm$), produced with a deionization apparatus (PureLab) from USF.

Dialysis membranes with a cut off of 12,000 Daltons, were purchased from Medicell, Italy.

Methods

Synthesis of PVA Coated Microbubbles

Synthesis of telechelic PVA microbubbles was described in the literature.[5,6] Stable (air-filled) PVA coated microbubbles were prepared by crosslinking telechelic PVA at the water/air interface. Vigorous stirring at room temperature for 3 hours of 2% telechelic PVA aqueous solution (100 mL) at pH 2.50 by an Ultra-Turrax T-25 at 8,000 rpm equipped with a Teflon coated tip, generated a fine foam of telechelic PVA acting both as colloidal stabilizer and as air bubble coating agent. The crosslinking reaction was carried out at room temperature and at 5 °C by adding hydrochloric or sulphuric acid as catalyst and stopped by neutralizing the mixture. Floating microbubbles were separated from solid debris and extensively dialyzed against Milli-Q water. An aqueous suspension of microbubbles was obtained and used for further chemical modification.

Preparation of β-Cyclodextrin Functionalised Microbubbles

Covalent linkage of β-cyclodextrin to microbubbles was achieved by acetalization reaction between microbubbles suspension (1 mg/mL) and a 0.02 M β-cyclodextrin

aqueous solution. After incubation for 24 h at 25 °C and pH 3 the resulting mixture was neutralized and extensively dialyzed against water in order to remove free β-cyclodextrin.

Preparation of Poly(L-lysine) Functionalised Microbubbles

Poly(L-lysine), PLL, was linked to microbubbles by imine linkages or by reductive amination procedure. 10 mL of aqueous suspension of microbubbles (1 mg/mL) were incubated with 3 g/L solution of poly(L-lysine) (MW 7,500) at room temperature and pH 5. Schiff base reduction was carried out by $NaBH_3CN$. After 24 hours, microbubbles were extensively dialyzed to remove unreacted poly(L-lysine). A dialyzed suspension of poly(L-lysine) coupled to microbubbles was concentrated by recovering the floating microparticles and used for spectroscopic investigations.

Preparation of Chitosan Functionalised Microbubbles

Chitosan was conjugated to microbubbles by using reductive amination procedure. Chitosan was dissolved in sodium acetate 0.2 M/acetic acid 0.3 M buffer at pH 4.5 to a concentration of 3.3 % (w/v). 5 mL of 2 mg/mL of microbubbles aqueous suspensions were added with 0.8 mL of chitosan solution at room temperature. The pH was carefully adjusted to 5.0 with acetate buffer, following the addition of $Na(CN)BH_3$. The resulting suspension was stirred for 5 days at room temperature. Microbubbles suspension was then extensively dialyzed and washed.

Characterization of Unmodified and Derivatized PVA Microbubbles

Particle size distribution was evaluated by dynamic light scattering, DLS, and laser scanning confocal microscopy. Details of these characterization methods have been already reported.[6]

FITC and RBITC were used for fluorescent labeling of the microbubbles. Fluorescent dyes at a typical concentration of 10 μM were added in a molar ratio of about 10^{-3} with respect to polymer repeating units into the suspension. Floating particles were washed by re-suspending them in Milli-Q water several times. Confocal images were collected by a confocal laser scanning microscope (CLSM), Nikon PCM 2000 (Nikon Instruments): a compact laser scanning microscope based on a galvanometer point-scanning mechanism, a single pinhole optical path and a multi-excitation module equipped with Spectra Physics Arion laser (488 nm) and He-Ne laser (543.5 nm) sources. A 60×/1.4 oil immersion objective was used for the observations. Tests carried out by measuring giant liposome shells provided a value of 0.25 μm for instrumental resolution. Shell thickness of fluorescent labeled microparticles were measured as the full width at half maximum of the fluorescence intensity profile.

Circular dichroism, CD, spectra typically in the range 210–300 nm were recorded by using quartz cell (1–10 mm) with JASCO J600 spectrometer equipped with a thermoregulated cell compartment to check the microbubbles surface modification by poly(L-lysine) and to observe peptide conformational behavior as a function of pH. Aqueous microbubbles suspension (1 mg/mL) was only slightly turbid and CD spectrum was recorded using untreated microbubbles suspension as a blank to determine the absence of artifacts in the CD spectrum of microballoon suspension. Only after this check a quantitative estimation of bound poly(L-lysine) concentration was attempted.

High resolution NMR ^1H spectra were recorded on a Bruker 400 MHz on D_2O suspension of microbubbles to check the conjugation of β-cyclodextrin to the microbubbles surface.

Isothermal microcalorimetry titration of β-cyclodextrin anchored microbubbles with amantadine hydrochloride allowed the determination of both the extent of microbubbles grafting and binding constant of the inclusion complex. Binding measurements were carried out with an isothermal microcalorimeter, TAM (Thermometrics), at 25 °C by adding 10 μl of amantadine

solution (0.015 M) to 2 mL of concentrated microbubbles aqueous suspension placed in a stainless cell. Binding constant determination of free β-cyclodextrin-amantadine complex in solution was carried out as well. Potentiometric titration of glucosamine residues of chitosan was used for quantitatively estimation of chitosan conjugation to microbubbles.

Cell Lines and Cell Growth Assay

LoVo and HT29 human colon adenocarcinoma cell lines obtained from American Type Culture Collection were used for citotoxicity tests. According to growth profiles preliminarly defined for each cell model, appropriate numbers of cells in the logarithmic growth phase were plated in each well of a 6-well plate and allowed to attach to the plastic surface for 24 h. Cells were then exposed to increasing volumes of 1 g/L PVA microbubbles suspensions for 7 days at 37 °C in a 5% CO_2 humidified atmosphere in air. At the end of incubation, cells were counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). Each experimental point was run in triplicate.

Results and Discussion

Characterization of the PVA Microbubbles and Microcapsules

Dimensional characterization in terms of average particle diameter and shell thickness have been carried out by coupling two different approaches, i.e. confocal scanning laser microscopy(CLSM) and dynamic laser light scattering (DLS). As an example, in Figure 1 is reported the scanning of the focused equatorial frame of a sample of rhodamine labeled microballoon shells and the fluorescence intensity profile, measured across the microparticle. The dimensional characteristics of the circled particle allowed an evaluation of the diameter of 5.2 μm and a shell thickness of 0.9 μm, estimated from the fluorescent contour showed in the insert. A statistically significant number of

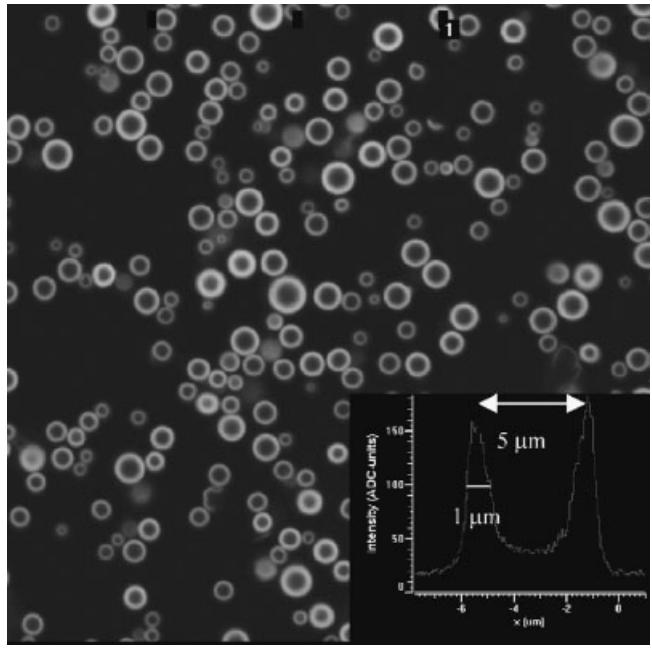

Figure 1.
2D CLSM image of rhodamine isothiocyanate labeled microbubbles prepared at room temperature. Insert: fluorescence intensity profile acquired for the size and shell thickness determination.

Table 1.
Dimensional parameters of PVA based microbubbles and microcapsules.

	External diameter, μm	Internal diameter, μm	Shell thickness, μm
Microbubbles	5[a] ± 1 5[b] ± 1	3[a] ± 1	0.90[a] ± 0.25[c]
Microcapsules	6[a] ± 1 7[b] ± 1	4[a] ± 1	1.00[a] ± 0.25[c]

[a] Confocal laser scanning microscopy.
[b] Dynamic light scattering.
[c] Confocal microscope resolution.

observations was in good agreement with DLS results.

Preliminary observations on microbubbles response to ultrasound show a peak in the ultrasound reflectivity at about 4 MHz, a typical working frequency of biomedical echography hardware.

An interesting feature of this system, useful for loading the core with a drug molecule and for using microbubbles as drug delivery system, is the conversion to microcapsules upon water ↔ DMSO exchange. The result of this solvent exchange is the air replacement with a liquid core with the obtainment of slightly larger microparticles with an average external diameter of 6.5 μm and a shell thickness of 1 μm.

Structural parameters of PVA microbubbles and microcapsules obtained at room temperature are summarized in Table 1.

Microballons Surface Decoration

The focus is on the biological/biomedical relevance of the coupling. Here we will report on some case examples of microbubbles surface decoration, the conjugation of β-cyclodextrins on the external interface of the PVA microbubbles. With this coupling process we have essayed the possibility of conjugation via acetalization. The cross-linking reaction leading to shell formation leaves unreacted several acetals at the shell surface. This moiety can be activated by lowering the medium pH followed by the coupling (acetalization) with the hydroxyl groups of β-cyclodextrins. Successful attachment was evidenced by the ^{1}H NMR spectrum showing the characteristic peaks of cyclodextrin moiety.

Such decorated microbubbles can function as host-carrier for a hydrophobic guest drug molecules accommodating in the internal cavity of the saccharide cyclic moiety. Binding capacity of β-cyclodextrin decorated shells has been tested with amantadine, a molecule with known hydrophobic features. Apparent equilibrium constant, K, and enthalpy change, ΔH, of the host-guest complex is showed in Figure 2.

Anti-tumour drugs, as doxorubicin, included in the cyclodextrin molecules provided evidence for a controlled release of the pharmacoactive molecules in treated human colon adenocarcinoma cell lines, LoVo, and HT29.[10]

Biocompatibility Assay

We evaluated the effect of long-term (7 days) exposure to increasing concentration of PVA microbubbles on the in vitro cell growth of two human colon adenocarcinoma cell lines,

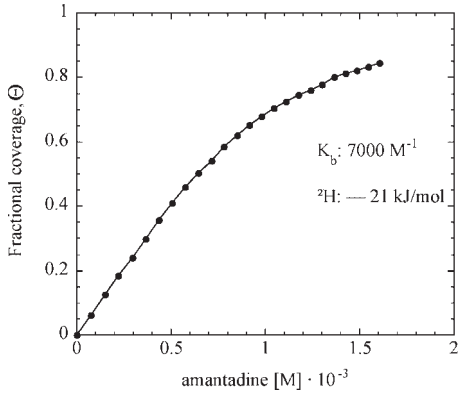

Figure 2.
Binding isotherm of amantadine to microbubbles anchored b-cyclodextrin at room temperature; continuous line non-linear best fit.

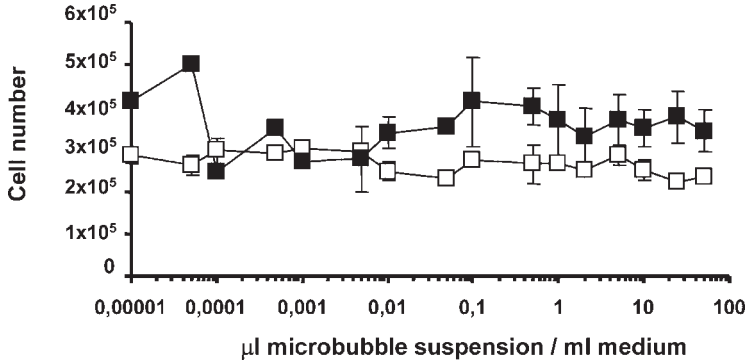

Figure 3.
Growth of human colon adenocarcinoma cell lines, LoVo (■) and HT29 (□), exposed to different volumes (from 0 to 50 µl/mL culture medium) of PVA microbubbles after 7 days. Data represents mean values (±SD) of three independent experiments.

LoVo and HT29, as shown in Figure 4. No significant antiproliferative effect was observed in cells exposed to microbubbles compared to control cells in both experimental models.

The presence of masked aldehydes in the form of stable intramolecular hemiacetals at physiological pH explains microbubbles biocompatibility towards several cellular strains as reactive aldehydes would jeopardize this property as they are known to be cytotoxic.

As far as the morphology of the cells is concerned, cells examined under a phase-contrast microscope displayed analogous shapes as compared to control cells. These findings indicate that PVA microbubbles do not interfere with tumour cell growth and can be considered a convenient support for the development of new drug delivery systems.

Chemical reactivity of microbubbles surface was tested by using both low and high molecular weight molecules. For the former type of molecules, functionalization was qualitatively assessed by spectroscopic methods, whereas a quantitative evaluation of the microbubbles derivatization with β-cyclodextrin, poly (L-lysine), and chitosan was determined.

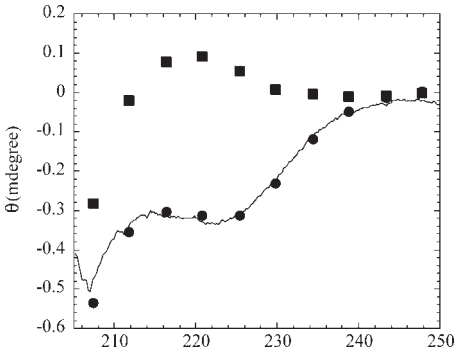

Figure 4.
Room temperature CD spectra of poly (L-lysine) anchored on PVA microbubbles via reductive amination: pH 7 (■); pH 12 (●), full line represents the best fit of the linear combination of the PLL CD spectra at low and high pH, i.e. random coil and α helix, respectively.

Poly (L-lysine) Surface Microbubbles Decoration

Modern therapeutic approaches use macromolecular assemblies to penetrate cellular membranes and ultimately introduce gene sequences.[11–13] DNA recognition by helical peptides containing lysine residues by formation of a macromolecular complex with the capability to be endocytosed by cells has been reported in the literature.[14,15]

In this respect we have tested the possibility to decorate the external surface of PVA based microbubbles with poly (L-lysine), PLL, and to investigate on the conformational properties of the anchored polypeptide. PLL conjugation on polymeric shell was carried out by Schiff base formation using the presence of the acetals moiety on the microballoon shell and of the

amino groups of poly (L-lysine). The total amount of tethered polypeptide was 81 mg lysine residue/g microbubbles corresponding to 67 mg/m^2, based on a specific surface area of microbubbles of 1.2 m^2/g (see Table 1) and molar ellipticity of PLL in random coil conformation measured by the circular dichroism. Reductive amination of the Schiff bases increases the PLL amount on the microballoon surface to about 550 mg/m^2. These payloads are higher than the protein and PLL contents, evaluated in about 1 to 3 mg/m^2, obtained at the oil/water interfaces[16] and at the PLGA surface microparticles respectively.

Moreover the chemical stability of the poly (L-lysine) tethered microbubbles was greatly improved by the reductive amination step. Tethered PLL by Schiff base conjugation was hydrolyzed in few weeks, whereas the reductive amination yielded an amine linkage resulting in a product stable for few months.

The conformational properties of poly (L-lysine) immobilized on the microbubbles shell, were investigated in order to understand the potentiality of these devices as carriers able to retain the conformational status of the attached macromolecule. It is known the ability of polypeptides to undergo a α-helix $\leftrightarrow$ random coil transition by varying the solution paramenters as pH, temperature, solvent polarity.[17] The conformational features of PLL anchored to PVA based microbubbles were tested by circular dichroism spectroscopy, an assessed approach for investigating the conformational state of polypeptides. α-helix content of polypeptide was evaluated by the method of Greenfield and Fasman.[17] Setting to 100% the α-helix content of the low molecular weight PLL, about 72 % of the polypeptide anchored as Schiff base or as amine was in the ordered conformation, according to the analysis of the CD spectrum of a suspension of PLL decorated microbubbles shown in Figure 4.

Chitosan Surface Microbubbles Decoration

Chitosan has the special feature of adhering to mucosal surface and improve the bioadhesion.[18] For this reason a variety of chitosan-based colloidal delivery carriers have been described for association and delivery of macromolecular compounds such as peptides, oligonucleotides and genes. Because of the high molecular weight of chitosan the extent of polysaccharides covalently bound to microbubbles, determined by potentiometric titration of microbubbles suspension, was very high, approximately 20 % g glucosamine/g microbubbles. The high yields of functionalization was also confirmed by confocal microscopy images, Figure 5, where chitosan aggregates surrounding microbubbles was also evidenced by FITC labeling.

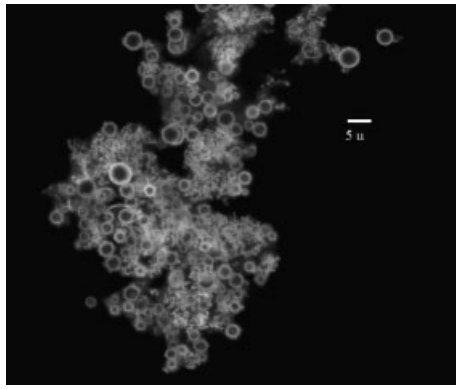

Figure 5.
CLSM of chitosan coated microbubbles.

Concluding Remarks

Ultrasound imaging is an important technique in diagnostics. Efforts have been reported in the recent literature to add multifunctionality to this approach[19–20] in order to use ultrasound imaging also as therapeutic tool. To this aim a new design of the ultrasound enhancing device has to be formulated. Although several drawbacks are still present in commercially available microbubbles, as broadness of the size distribution, short lifetime, not complete biocompatibility, the use of injectable lipidic microbubbles as ultrasound contrast agent has opened a diagnostic/therapeutic route in the echographic

methodologies. We are exploring the formulation of polymeric microbubbles working as echogenic systems and controlled drug delivery systems that may solve some of the weaknesses displayed by the present devices. The multifunctionality resides in the possibility to tailor the physical and chemical characteristics of the injected microballoon shell and to deliver the bound payload upon sonic irradiation. In this way specificity, bioavailability and visualization of the target tissue/organ can be optimized in this non-invasive approach.

The results obtained in this study show that PVA microbubbles offer some remarkable advantages as long shelf life, viability, limited size polydispersity, versatility in the tethering of many biological relevant drug molecules.

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