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# Comprehensive characterization of elastomeric Polyhydroxyalkanoate and its sensor applications

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#### 44 1. Introduction

45 More than 320 million tons of plastics generated per year represent a serious environmental problem 46 and potential hazard [1]. Biopolymers, particularly polyhydroxyalkanoates (PHAs), are promising alternatives to a slow- or nondegradable petroleum-based plastics [2]. PHAs are biopolyesters that have 47 48 been applied in many sectors of modern science and industry. PHAs are biocompatible and 49 biodegradable and as such have a positive social and environmental dimension [3]. Degradation metabolites of PHAs are not toxic; they degrade into carbon dioxide and water (under aerobic 50 conditions), or carbon dioxide and methane (under anaerobic conditions) [4]. In 2007, the Food and 51 52 Drug Administration (FDA) approved the use of P(4HB) for surgical sutures in clinical applications 53 and from that period medical applications of PHAs have increased [5]. Examples of these applications 54 include: scaffold for heart valve and cartilage tissue, micro- and nanospheres and antibacterial coating 55 material [6], [7]. PHAs are mainly produced by biotechnological (fermentative) methods. More than 56 150 PHA subunits have been identified to date [8] and their structure determines mechanical properties 57 and degradation rate [9]. PHAs can be degraded by the activity of microorganisms (bacteria and fungi), 58 but also by enzymes in bovine serum, pancreatin and synthetic gastric juice [10]. The carbon sources 59 and the metabolic potential of the microorganisms involved in their synthesis have an influence on PHA chemical properties [11]. The molecular mass of PHA is dependent on growth conditions such as carbon 60 61 source, culture medium or fermentation mode [12]. PHAs are insoluble in water, but soluble in chloroform and demonstrated a good resistance to moisture and UV light [13]. The composition of the 62 monomeric unit of PHAs has an influence on their mechanical and temperature characteristics with 63 short chain length (scl) PHAs being hard, brittle polymers while medium chain length (mcl) PHAs are 64 65 softer, more elastomeric polymers [14]. The mechanical properties of PHAs can be improved by means of an electrospinning technique [15]. Combining PHAs with other polymers can improve their 66 flexibility, which is an important parameter for medical applications [16]. The melting temperature of 67 scl-PHA is above 140°C, while melting temperatures of mcl-PHA is usually between 40 and 60°C, and 68 69 above this range they are amorphous and sticky. However, melting temperatures are highly dependent 70 on the monomer composition, thus mcl-PHA homopolymers can have melting temperatures even higher than 60°C [16, 17]. The applications of PHAs as biodegradable polymers is manifold and it will be even 71 72 higher if their mechanical and hydrophobic properties can be modified and fine-tuned with respect to 73 the requirements of the specific application [18]. Aligning fibers in the internal structure of PHA, achieved by the electrospinning technique, can improve the mechanical characteristics compared with 74 75 randomly oriented fibers inside the PHA structure [19]. Furthermore, the wettability and the contact 76 angle of polyhydroxyalkanoates electrospun membranes can also be adjusted by changing the polymer 77 concentration [20].

78 An analysis of the literature reveals that the majority of published papers have considered (bio)medical applications of PHAs, but there is a lack of articles reporting sensor applications of PHAs. Previously, 79 80 PHB film was reported to be suitable as a membrane for the incorporation of haemoglobin to boost the electron transfer rate of this protein. Further modifications using pyrolytic graphite electrodes and/or 81 the addition of peroxidises paved the way towards the application of PHB-embedded proteins as an 82 integral part of H<sub>2</sub>O<sub>2</sub> biosensors [21], [22]. Only one more biosensor application was found in the 83 84 literature; another type of PHA (biopolymer containing 3-hydroxyvalerate (3HV), 5-hydroxydecenoate (5HDE) and 3-hydroxyoctadecenoate (3HODE)) was used to develop of a hybrid nanocomposite based 85 biosensor containing gold nanoparticles, horse radish peroxidase and PHA/AuNP/HRP/ITO for the 86 87 quantitative detection of artemisinin in body fluids [23].

This study aims to expand the application niche of PHA based sensors. Herein we analyse the structural,
morphological and mechanical properties of a biotechnologically produced PHA sample, more
specifically a medium chain length PHA (mcl-PHA). The inductive-capacitive (LC) resonant circuit

91 structure was constructed using this sample. This device was applied for the remote detection of liquid

solution in which the sensor was immersed (such as artificial saliva or simulated gastric fluid). Using

- 93 the principles of wireless coupling we have successfully detected the chosen fluid by measuring the
- 94 shift in the resonant frequency of the LC structure fabricated on a PHA substrate covered with gold as95 a conductive material.
- 96

# 97 2. Materials and Methods

# 98 2.1 Production method of PHA sample

99 Mcl-PHA biopolymer, specifically polyhydroxyoctanoate (PHO; contained > 95% 3-hydroxyoctanoic 100 acid as the monomer), was produced via fermentation using Pseudomonas putida KT2440 strain by Bioplastech Ltd., using basic fermentation conditions as described previously [24] and the octanoic acid 101 as substrate [25]. The PHO content of cells was determined by methanolysis of 10 to 15 mg of 102 lyophilized cells in the presence of 15% (vol/vol) sulfuric acid. The resulting methyl esters of the 103 constituent hydroxyalkanoic acids were analyzed by gas chromatography. For identification of the 104 methylesters, gas chromatography-tandem mass spectrometry (GC-MS-MS) was also performed. The 105 remaining monomers were trace amounts of 3-hydroxyhexanoate and 3-hydroxydecanoate. Mcl-PHA 106 107 films were prepared by the solvent casting method. Initially, PHA (2 g) was dissolved in acetone (10 108 mL) at 25 °C, after which it was poured into a glass Petri dish and left to dry in the air at ambient temperature for 10 days. 109

# 110 2.2 Characterization methods

A 3D optical Profilometer (Huvitz microscope with Panasis software) and scanning electron 111 microscopy (SEM, Hitachi TM3030) were used for structural and surface analysis. Morphology, 112 roughness and frictional properties of the sample were studied with an atomic force microscope 113 AutoProbe CP-Research SPM (TM Microscopes-Bruker) using a 90 µm large area scanner. The 114 115 measurements were performed in contact mode using Bruker Phosphorous (n)-doped silicon contact metrology probes, model MPP-31123-10 with Al reflective coating and symmetric tip. AFM images of 116 topography, "error signal" and Lateral Force Microscopy (LFM) signal were taken and later analyzed 117 using two software packages, Image Processing and Data Analysis Version 2.1.15 and SPMLab 118 Analysis, DI SPMLab NT Ver. 6.0.2. The contact angle method was used for wettability analysis. The 119 contact angle measurement was performed in an optical laboratory located in a clean room class ISO 8, 120 nitrogen and air class ISO 5, vacuum. A Bausch & Lomb MicroZoom Microscope connected to a laptop 121 with software tool ISCapture were used for photography. Laboratory Power Supply Manson NSP 3630 122 123 provided the necessary light for measurement. For mechanical characterization, a nanoindentation method was used with a G200 nanoindenter, having a Berkovich diamond tip. A Vector Network 124 Analyzer E5071B was used for measuring S-parameters of LC structure, in different media. 125

# 126 2.3 Preparation of test fluids

127Two different fluids were prepared in order to test functionality of the fabricated structure: (a) artificial128saliva and (b) simulated gastric fluid. The artificial saliva had the following composition: Sodium129carboxymethylcellulose - 10 g/l, Sorbitol - 29.95 g/l, Methyl p-hydroxybenzoate - 1.00 g/l, Sodium130chloride - 0.87 g/l, Di-potassium hydrogen orthophosphate - 0.80 g/l, Potassium chloride - 0.22 g/l and131Lemon aroma - 5 ml. The simulated gastric fluid, without pepsin, was prepared as mixture of 0.2%132(w/v) Sodium Chloride in 0.7% (v/v) Hydrochloric Acid adjusted to pH equal to 1.2 [26].

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# 134 3. Results and Discussion

- 135 *3.1 Structural, morphological and wettability properties of PHA sample*
- 136 *3.1.1. Structural characterization*

137 In the last few years, PHAs have been studied to be used in tissue engineering for hard and soft tissue replacement, and as therapeutic delivery carriers [27]. For such applications mostly scl-PHAs, such as 138 polyhydroxybutyrate (PHB) and its copolymers have been applied. 139

140 In this study, polyhydroxyoctanoate (PHO), representative of the mcl-PHA family, was evaluated for biosensor applications due to its specific thermo-elastomeric characteristics. Processed mcl-PHA film 141 sample is shown in Fig. 1(a). Structural characterization was performed by SEM to visualise the surface 142 143 and the cross section of the PHA sample. An SEM micrograph of the polymer surface with a magnification of 200 is presented in Fig. 1(b), while a cross section of the sample with magnification 144 of 250 times is depicted in Fig. 1(c). Speckles of dust are evident on the surface of the film (Fig. 1(b)) 145 146 while the side of the film that was in contact with glass during the solvent casting appears denser, as 147 the cross section revealed (Fig. 1(c)).

148 149

#### Figure 1.

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A 3D Optical profilometer was used to determine the exact thickness of the PHA polymer. The role of 151 the profilometer was to provide 2D and 3D insight into topographic surfaces, and was used for micro-152 and nano-level measurements. Due to the transparency of the sample, the surface of the polymer was 153 154 coloured purple with a marker, prior to starting measurement with the 3D Profilometer. The purple dye did not affect the measurement parameters or the performance of the device. The 2D image obtained is 155 presented in Fig. 2(a) with magnification of  $\times 20$ , and the 3D image is presented in Fig. 2(b). The 3D 156 profile shows the thickness of the sample, which was estimated to be around 300 µm. The polymer was 157 158 not of uniform thickness across the whole surface, and varied in the range  $\pm 10 \ \mu m$ .

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- Figure 2.
- 3.1.2. Morphological characterization

163 Selected results of AFM characterization are presented in Figs. 3 and 4. The 3D topography of the sample is shown in Fig. 3 for the two scanning areas, (a) 50  $\mu$ m × 50  $\mu$ m, and (b) 5  $\mu$ m × 5  $\mu$ m. For 164 165 these areas, RMS roughness of the sample was 608 nm and 73 nm, respectively. Results of LFM 166 measurements are shown in Fig. 4. For the mentioned scanning areas, topography and LFM images are shown in parallel. By analyzing these images we can conclude that the sample surface is heterogeneous 167 regarding frictional properties. The darker the region on the LFM image, the higher the value of the 168 friction coefficient. In other words, the darker regions are "stickier" than the rest of the sample. 169

Figure 3.

Figure 4.

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PHA films or membranes can be produced by various techniques that include compression-molding, 175 solvent-casting, or electrospinning, and more recently 3D printing fabrication [28]. It has been shown 176 177 previously that solvent-casted films have higher roughness in comparison to the compression-molded 178 PHA membranes, however that did not affect the adherence of cerebellar granule neurons [29].

#### 179 3.1.3. Characterization of wettability

For this purpose, we used the contact angle method where the static contact angle of a droplet on a
polymer was measured over a period of time until the droplet (about 2 µl) was absorbed or spilled over
the surface. The droplet images were analyzed by ImageJ software (option drop\_analysis) depicted in
Fig. 5.

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#### Figure 5.

Fig. 5(b) illustrates the change of contact angle for a drop of distilled water over a period of 10 minutes. The values of the contact angle over the entire measurement period, to which the polymer was subjected, are below 90°, which categorize this polymer as hydrophilic, which means with higher wettability. An increase in the number of polar groups can make polymers more hydrophilic. This property is favorable for PHA sensor applications such as the LC sensor described in this paper, because it ensures that the liquid medium has better contact with the substrate.

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#### 192 *3.2 Mechanical properties of PHA sample*

One of the most important material properties is its mechanical endurance, which shows how much load (force, moment) a structure fabricated of the specific material can resist. Multiple nanoindentation tests were carried out with sets of ten indentations to ensure the credibility of the measured results. A preset depth of 5  $\mu$ m was set, while the reaching load time was set to 15 s, and the peak loading time was set to 5 s. Nanoindentation testing was performed in three cases: (1) PHA sample; (2) PHA sample after incubation in artificial saliva for twenty-four hours, and (3) PHA sample after immersing in a simulated gastric fluid for twenty-four hours. The results obtained are depicted in Fig. 6.

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#### Figure 6.

201 Fig. 6 shows the mean load-displacement curves for a maximum preset depth of 5  $\mu$ m, where the penetration loads were around 2.7 mN for pure PHA, 2.5 mN for the polymer in saliva, and 2 mN for 202 203 the polymer in gastric acid. It can be seen from Fig. 6 that the displacement into the surface exceeds the 204 preset depth of 5  $\mu$ m by a few nanometers, due to the hold time of 5 s on the preset depth so the tip enters a few nanometers more into the studied material. The obtained results confirmed theoretical 205 206 expectations; the pure polymer demonstrated the highest resistance to mechanical stress. When the polymer was in saliva, this fluid reduced its mechanical properties, softening the material. The polymer 207 208 that was immersed in the gastric fluid exhibited the lowest resistance to mechanical force. This is due to gastric fluid being an acid with pH around 1.2, which leads to degradation of the polymer and the 209 breaking of double bonds within the polymer structure. The measured values of Young's modulus were 210 211 58.8 MPa in the case of pure PHA sample, 49.1 MPa for PHA in the artificial saliva and 38.1 MPa for PHA in the gastric fluid, respectively. The hardness value was 8 MPa for the PHA sample, 7 MPa for 212 PHA in the artificial saliva and 5 MPa for PHA in the gastric fluid. These differences in elastic modulus 213 214 and hardness values are a consequence of the exposure of the PHA sample to fluids that affected its elasticity and strength. The obtained values of the studied parameters are in good agreement with the 215 216 data available in the literature. In [30] Young's modulus had a maximum of 25.4, 14.1, and 12.6 MPa 217 for polyhydroxyoctanoate (PHO) films obtained from ethyl acetate, acetone, and chloroform solution, respectively. These values are comparable with the values for Young's modulus presented in our study, 218 however, in [30], authors used an additional step for PHO film purification, which is one of the reason 219 to consider our approach more eco-friendly. In our study, the ratio "displacement/thickness of the PHA 220 221 film" was very low (around 0.016), meaning that nanoindentation tip will reach only the superficial 222 layer of PHA. However, a thinner polymer film will have higher Young's modulus values, more 223 precisely higher values of displacement/thickness ratio will result in an increase of Young's modulus,

which is caused by the substrate effect.

225 Since the sensor proved to be a fully functional device, several material characterization methods were applied in order to assess the quality of the solvent-casted mcl-PHA film itself as well as to analyse 226 structural, morphological, mechanical and wettability properties, which are essential for the underlying 227 operation principle and field of application of the sensor. SEM analysis was used to detect density of 228 229 the polymer as a substrate of the developed sensor. 3D profilometer was used to determine the thickness 230 of the substrate, because sensor properties are dependent on this thickness. AFM analysis was implemented to determine the roughness of used PHA film, because this surface property influences 231 232 how applied material or media will have a good adherence on the surface of the structure. Most biosensor applications for PHA polymers would be within a liquid environment, thus wettability was 233 234 also studied. One of the intended applications of the proposed structure is in the field of the selfdegradable sensors in the media in our body. Because of that, we also analyzed mechanical properties 235 236 or how the presented sensor softens up when exposed to saliva or gastric fluid.

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#### 238 *3.3 Sensor applications*

We designed an inductor-capacitor (LC) structure, composed of an interdigitated capacitor with 5 pairs 239 240 of fingers (electrodes) and a helically wound inductor. Uniquely, both parts (components) are planar 241 and realized in one layer, which means there is no overpass or underpass such as would usually be used in spiral inductor design. The width of the electrodes and the gap between them were both equal to 600 242 243  $\mu$ m. The total dimension of the LC structure was 30 mm  $\times$  12 mm. The LC sensor was fabricated using a cutting plotter machine, curving the structure in the PHA substrate (Fig. 7(a)). After that, gold was 244 vapored onto this structure, as can be seen in Fig. 7(b), in order to obtain the conductive structure at the 245 246 top. Nominal capacitance of the inderdigitated part of the structure was around 10.76 pF, whereas the 247 inductance of the inductive part of the structure was approximately 1.53 nH.

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#### Figure 7.

- The Vector Network Analyzer (VNA) was used to measure the electrical characteristics of the sensoras it is depicted in Fig. 8, using a copper antenna coil.
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### Figure 8.

The S11-parameter of the antenna was measured using VNA and it was subtracted from the measured value of the S11-parameter obtained for the PHA-based LC structure exposed to different media. This eliminated any error introduced by the antenna itself. The first measurement was conducted under conditions such that the air was the dielectric material between the fingers of the interdigitated capacitor. The second and third measurement cycles were performed with artificial saliva or artificial gastric fluid, respectively, between the electrodes of the capacitor. The measured amplitude of the S11-parameter as a function of frequency is presented in Fig. 9, for the three above-mentioned media.

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#### Figure 9.

It can be concluded from Fig. 9, that not only a shift in the resonant frequency was obtained when the sensor was exposed to different media, but also the magnitude of the S11 parameter increased in the liquid medium. The sensitivity of the proposed sensors can be expressed by  $\Delta S_{11}/\Delta f_{res}$  and the value of 0.3 dB/MHz was obtained. Figure 10 demonstrates a shift in the resonant frequency of LC structure towards lower values. The value and change in the resonant frequency is determined by the type of dielectric located between the fingers of the interdigitated capacitor of the LC resonant circuit structure. Depending on the value of the relative permittivity of the material placed between the capacitor 269 electrodes, the capacitance of the capacitor in the LC structure varies. Capacitance has the lowest value in air while it increases after exposure to the artificial saliva, and even more after immersion in the 270 artificial stomach acid. The resonant frequency is inversely proportional to this capacitance, based on 271 the equation  $f_{res} = 1/2 \cdot \pi \sqrt{L} \cdot C$ . The studied media, artificial saliva and simulated gastric juice, have some 272 similarities (containing water, mucus, salt), but also some differences (presence of iodide, pertechnetate, 273 and bromide). These differences lead to different values of their relative permittivity. The difference in 274 275 the dielectric constant was used to change capacitance of the capacitive part of the proposed sensor structure and consequently its resonant frequency. By means of the antenna coil, this shift in the 276 resonant frequency can be monitored remotely, without contamination of the analysed media. 277 Measuring S-parameters, using Vector Network Analyzer E5071B, we can determine both amplitude 278 of S11-parameter and  $f_{res}$  in different media. This instrument was chosen as it enabled application of 279 wireless (using antenna) and non-contact measuring principles. The qualitative advantages of this 280 281 approach are as follows: (a) the terminals (wires) are not necessary from the component under test; (b) the contamination effects are eliminated; (c) the applied method is non-destructive for the tested 282 283 component.

#### Figure 10.

285 Figs. 9 and 10 demonstrate that the highest resonant frequency value of the studied PHA-based LC structure was reached in air, being equal to 1.24 GHz. Resonant frequency drops to 1.23 GHz when the 286 287 artificial saliva was used as the dielectric. The lowest value of the resonant frequency in the LC structure 288 was observed when artificial gastric fluid was used as the dielectric, being equal to 1.22 GHz. Thus, a shift in the resonant frequency of 10 MHz per new medium was obtained, which can be also used as a 289 290 measurable indicator of the sensitivity of the proposed structure. To compare the sensitivity of the 291 proposed PHA-based sensor and other type of sensors from literature that also used resonant frequency as a measured quantity, Table 1 summarizes a comparison among different studies dealing with sensors 292 for detection of various parameters of the human body. From Table 1, quantitative benefits of the 293 proposed technique can be also noticed, because the difference in S11 amplitude and shift of resonant 294 295 frequency between the various media has high values (1 dB/5 dB and 10 MHz, respectively), which 296 enables clear distinction between them.

#### 297

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#### 298 Table 1

299 Comparison of the sensitivity of sensors proposed in this paper and other reported studies.

Paper	Detection parameter	Sensing principle	Sensitivity (shift in)	
			Resonant freq.	Amplitude  S11
This work	saliva, gastric fluid	wireless LC	10 MHz per new	1 dB for saliva
			medium	5 dB for gastric fl.
Ref. [31]	gastric pressure	inductive coupling LC	0.6 kHz / mmHg	n.a.
Ref. [32]	pH of liquid sample	planar inductor	4 MHz / pH	n.a.
Ref. [33]	glucose, galactose,	capacitive coupling	304 kHz, 540	0.01, 0.0125,
	fructose	split rings	kHz, 2.2 MHz / mg/dl	0.0075 dB / mg/dl
Ref. [34]	pH of liquid sample, such as gastric acid	capacitive sensor + VCO	0.35 MHz / pH	n.a.
Ref. [35]	ε <sub>r</sub> value (for saliva sample)	coplanar interdigital capacitor	129 MHz for $\epsilon_r$ from 60 to 70	n.a.
Ref. [36]	glucose concentration in fluid	liquid channel- loaded capacitor	2 MHz / mg/ml	n.a.
Ref. [37]	aqueous glucose	microwave	n.a.	1.2 dB from water
	concentration	dielectric resonator		to glucose conc. of 300 mg/ml

Ref. [38]	glucose concentration in tears	graphene-AgNW electrodes	n.a.	0.5 dB/M
Ref. [39]	glucose concentration in saliva	RF-trilayer sensor	0.6 MHz in 1 g $L^{-1}$ of glucose	1 dB for saliva
Ref. [40]	pseudomonas aeruginosa, staphylococcus aureus	Interdigitated capacitor on foil	0.25 MHz per dilutions of concentration	n.a.

301 Thus, it can be concluded that the PHA-based LC sensor structure can successfully detect different liquids to which the sensor can be exposed, through monitoring the shift in the resonant frequency. The 302 presented sensor can be calibrated using pH meter (we use pH-meter InoLab 720, WTW, Weilheim in 303 304 Oberbayern, Germany), bearing in mind that two studied media, saliva and gastric acid, differentiate 305 significantly in their pH values. Namely, saliva has pH value between 6.2 and 7.6, whereas gastric juice 306 has acidic pH value in the range from 1 to 2. Additionally, our measured results can be calibrated or confirmed by bulky and costly HPLC or LC/MS instruments. The presented technique based on the 307 wireless principle can find useful application in the biodegradable electronics domain. 308

309

# 310 4. Conclusions

311 This work demonstrated the successful implementation of PHA as a low-cost substrate for LC resonant sensors, which would increase the commercial competitiveness of these polymers. A comprehensive 312 313 structural, morphological and mechanical characterization of the solvent-casted mcl-PHA was performed. The sensor structures were tested in air, and liquid media including artificial saliva and 314 315 artificial stomach acid. The sensor described in this work, realized on a biodegradable, flexible 316 substrate, can find wide application in the fields of medical diagnostics and biomedical engineering. In 317 the field of biomedicine, biopolymers show many advantages that make them superior to synthetic polymers, predominantly because of their natural origin. In addition, the presented PHA sensor is 318 319 environmentally friendly, since there are no by-products during the fabrication process or from 320 degradation that would harm water, land, air or living things.

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# 440 Figure captions:

- 441
- 442 Figure 1. (a) Visual appearance of the processed PHA sample, (b) SEM micrograph of the sample at
  443 ×200 magnification, (c) SEM micrograph of the sample cross-section at ×250 magnification
- Figure 2. (a) 2D profilometer image of surface structure of sample; (b) 3D profilometer image of sample
- Figure 3. Three-dimensional AFM images of PHA sample topography obtained for two scanning areas: (a)  $50 \ \mu m \times 50 \ \mu m$  and (b)  $5 \ \mu m \times 5 \ \mu m$
- Figure 4. 2D AFM images showing topography ((a) and (c)) and LFM signal ((b) and (d)) for the scanned areas  $50 \ \mu m \times 50 \ \mu m$  and  $5 \ \mu m \times 5 \ \mu m$
- Figure 5. (a) Detail of the user interface of ImageJ software during the measurement of the contact angle(b) Graphical representation of contact angle results for a drop of distilled water
- 451 Figure 6. Load-displacement curves for three analyzed cases
- 452 Figure 7. LC resonant structure: (a) manufactured from PHA, (b) after gold coating
- 453 Figure 8. Sensor appearance when there is artificial saliva between the capacitor electrodes
- 454 Figure 9. Frequency dependence of amplitude of S11 parameters in three chosen media
- 455 Figure 10. Variation of the resonant frequency depending on the type of the medium
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