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EVALUATION OF BIOACTIVITY OF POLY(ACRYLIC ACID) – HYDROXYAPATITE – NANOGOLD COMPOSITES IN *IN VITRO* CONDITIONS

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In this paper we report the preparation and characterization of ceramic-polymer composites doped with gold nanoparticles. Properties and applications in medicine and dentistry of colloidal gold nanoparticles depend upon their size and shape. The influence of metallic nanoparticles on the *in vitro* behavior was investigated by pH analyses of simulated body fluid and artificial saliva. The nanocomposites were characterized with the use of X-ray Diffaction (XRD) and Fourier Transformed Infrared Spectroscopy (FT-IR) methods. The results of *in vitro* tests confirmed that it is possible to produce hydroxyapatite/polymer (HA/polymer) composites doped with gold nanoparticles (AuNPs) for medical applications. Investigations proved that presence of gold nanoparticles in composites had influence on *in vitro* behaviour of HA/Polymer/AuNPs composites in artificial physiological fluids. Research results indicate that obtained composites demonstrate bioactivity features and could become innovative materials for biomedical and dentist applications.

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Keywords: Hydroxyapatite, Poly(acrylic acid), Gold nanoparticles, incubation, SBF, artificial saliva

1. Introduction

Polymer materials currently used in implantology have unfavorable mechanical properties such as mechanical strength, Young's modulus and crack resistance. However, it is expected that composite materials might improve these defects of polymers. For example polymer composites (biostable and bioresorbable) with hydroxyapatite as the second phase in the form of particles or fibres offer the possibility of production of materials with controlled mechanical properties and assumed biological behavior [1-5]. Application of resorbable matrices leads to a multifunctional implants, in which after defect repair and resorption of the polymer, hydroxyapatite phase can be the scaffold for bone tissue growth [4-7]. In bone surgery the most frequently used bioresorbable polymers are caprolactam, polylactides and their copolymers with glycolide [8]. The addition of hydroxyapatite particles to biostable polymers reduces the durability of the composite in comparison with starting material. Composites with addition of hydroxyapatite exhibit favorable biological behavior by acting of bioactive particles (originating from HA) as anchors for bone tissue, which provides a good connection with the living tissue implants. In case of biostable composites introduction of particles or fibers to polymer matrix might change the mechanism of biological interaction with

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the environment and thus long-term functioning of implants. The most commonly used biostable polymers in bone surgery are: polyethylene, poly(ethylene oxide), poly(methyl methacrylate) [9-11]. It is important to assess the surface properties of implant materials in contact with fluids simulating internal environment of human body, such as SBF (artificial blood plasma), Ringer's solution or artificial saliva. The behavior of composites in the environment of a living organism depends on many factors including chemical and phase composition, crystalline phases, the shape and size of the crystals, as well as the presence of network defects [12-14].

In this paper we synthesized polymer/ceramic composites with addition of nanoparicles, which subsequently were treated with fluids simulating body environment. After incubation composites were characterized with X-ray Diffaction (XRD), Fourier Transformed Infrared Spectroscopy (FT-IR), Gel Permeation Chromatography (GPC) and pH analysis.

2. Experimental part

2.1. Methods

FT-IR analyses were carried out with the use of Scimitar Series FTS 2000 spectrophotometer produced by the Digilab company within the basic infrared range of 400-4000 cm^{-1}. The sample of 0.0007g was pressed with 0.2000±0.0011 g KBr into a pellet. The Deuterated Tri-Glycine Sulfate (DTGS) detector was used for mid-IR range measurements. There were 16 scans with the resolution of 4.

The phase composition of the samples was analysed with the use of X-ray diffraction with Philips X'Pert diffractometer equipped with a graphite monochromator PW 1752/00, Cu Kα 1.54 nm, Ni filter (40 kV, 30 mA).

The multifunction device CX-742 produced by the Elmetron company was applied in the pH analyses.

The weight and number average molecular weight of solution ingredients after degradation were measured by gel permeation chromatography GPC method at 35°C, relative to poly(ethylene glycol)/poly(ethylene oxide) standards on POLYSEP-GFC-P4000 column (Phenomenex) and a refractive index detector. The water with a flow rate of 0.5 ml/min was used as the eluent.

2.2. Materials

All chemicals were of reagent grade and used without further purification. Hydrogen tetrachloroaurate (III) trihydrate (0.21% solution), trisodium citrate, acrylic acid, ammonium persulfate and potassium hydroxide were purchased from POCh (Poland). Diacrylate ethylene glycol (M = 525) as crosslinking agent was purchased from Sigma Aldrich, however the solution of sodium acrylic copolymer Dispex N40 from Ciba Specialty.

2.2.1. Gold nanoparticles AuNPs

The gold nanoparticles were prepared by sodium citrate reduction method [15] with modifications. The synthetic method developed for this experiment consistently produces stable gold nanoparticles, provided that conditions are properly controlled [16]. $AuHCl_4$ was applied as a source of gold ions and trisodium citrate was used both as stabilizing and reducing agent.

2.2.2. Hydroxyapatite HA

Hydroxyapatite (HA) applied in our research was animal origin (pork) and was prepared in our original method. The process of obtaining hydroxyapatite consist of three stages:

1. Acid hydrolysis of pork bones using lactic acid under the pressure 0.3 MPa and at temperature 135°C.
2. Preliminary calcination at a temperature of 600^{o}C in a chamber kiln with electric heating in air atmosphere within 180 minutes.
3. In the last stage – proper calcination – the unified material from the second stage was calcined at 950^{o}C while kept at maximum temperature within 150 minutes in the chamber kiln [17].

2.2.3. Preparation of HA/polymer/AuNPs nanocomposites

Preparation process of ceramic/polymer composites modified with gold nanoparticles consists of following stages:

1. Neutralization of acrylic acid by 40% potassium hydroxide solution,
2. Preparation of HA dispersion in Dispex N40 (dispersing agent),
3. Polymerization and finally crosslinking of the product.

In connection with neutralization, which was exothermic reaction, the rapid increase of temperature was observed. Then the mixture was cooled and before prepared HA dispersion was added. Next, the proper amount (1-5%) of gold nanoparticles suspension (AuNPs) with concentration of 50 ppm, initiator and crosslinking agent were added. After homogenization the mixture was microwave irradiated for 3 minutes to enhance polymerization and crosslinking reactions [18-21].

3. Results and discussion

3.1. FT-IR analysis of composites

Spectral analyses were made for all ceramic-polymer composites doped with gold nanoparticles.

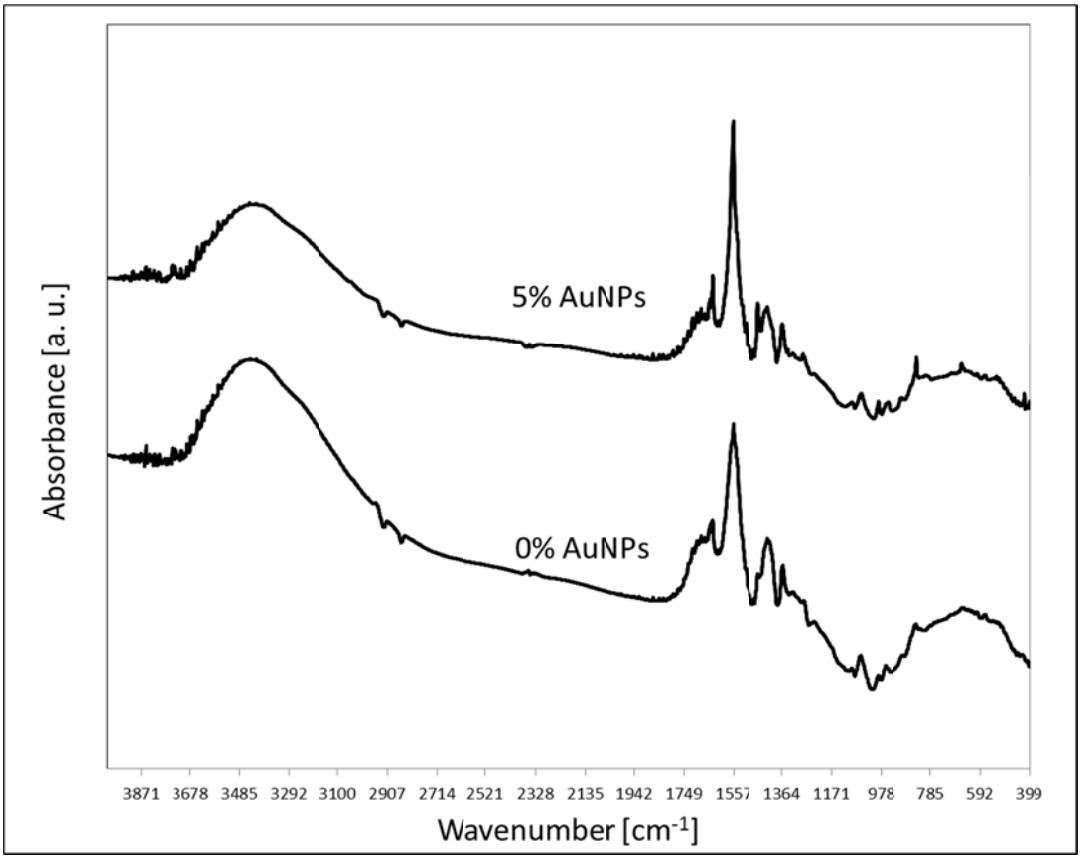

Fig. 1. FT-IR spectra of composites

Figure 1 demonstrates the FT-IR spectrum of composite with content of 5% AuNPs and sample without gold nanoparticles. All spectra of sample with AuNPs were similar. On the basis of these investigations it was found that vibration characteristic for functional groups present in polymer matrix, were observed. The broad absorption band at the range of 3500 – 3000 cm^{-1} corresponded to stretch vibrations of hydroxide group was observed - the band broadening was a result of water molecules absorbed into the polymer matrix. In the range of 2970 – 2930 cm^{-1} the observed valence vibrations were from the CH_2 group, however bands in the range of 1150 – 350 cm^{-1} came from torsional vibrations of this functional group. The absorption band at 1640 cm^{-1} was corresponded to tensile vibrations of C=O group. The band in the range of 1580 – 1475 cm^{-1} was a result of deforming vibration of –NH and C–N groups. However bands from CH_2-CO groups were visible in the range of 1435 – 1390 cm^{-1}. The bands in the range of 1100 – 1200 cm^{-1}

were corresponded to valence vibrations of –C–N– groups. No absorption bands from hydroxyapatite were observed. The lack of the most intensive band at 1200-1000 cm^{-1} was corresponded to asymmetric stretch vibrations of P-O bond, as well as absorption at 964 cm^{-1} was from symmetric vibrations of P-O bond. Vibrations of O-P-O bonds assigned to the absorption bands within wave numbers range of 604-563 cm^{-1} were not noticed. In the spectra of obtained composites the low intensive bands at 1457 and 881cm^{-1} corresponded to stretching vibrations of CO_3^{2-} were not observed. After comparing of these two spectra it was observed that peaks at 1551, 1439 and 814 cm^{-1} were strengthened and shifted to 1558, 1423, 835 cm^{-1} respectively.

3.2. XRD analysis of composites

The XRD analyses of ceramic/polymer composites modified with gold nanoparticles were carried out. Figure 2 demonstrates the typical XRD pattern. The amorphous polymer matrix on the XRD pattern was observed as "background noises" and it covered peaks came from hydroxyapatite (HA). The content of ceramic particles in composite materials amounted to 1% and it could be near the limit of phase determination by roentgenographic method.

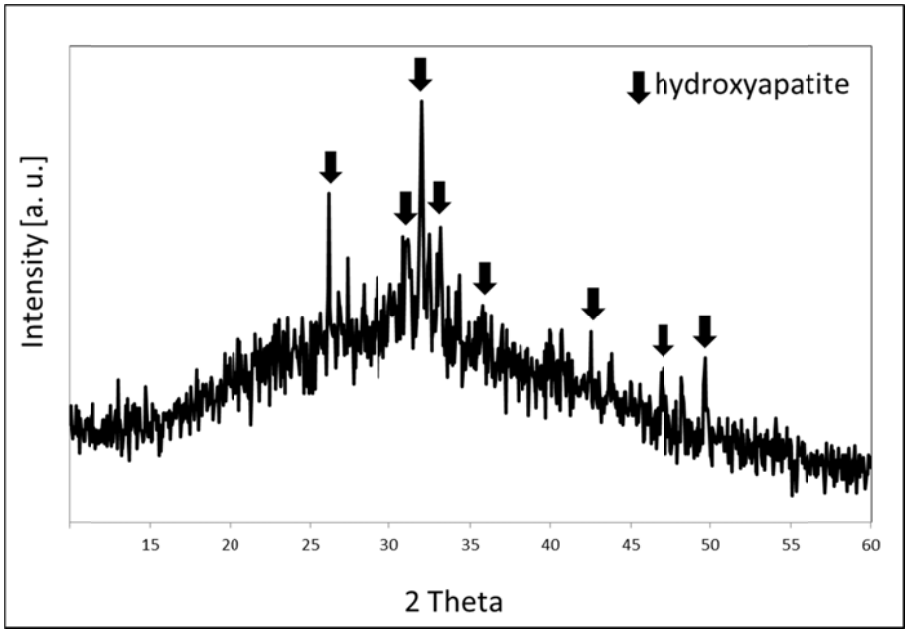

Fig. 2. Typical XRD pattern of ceramic/polymer composite with AuNPs

3.3. Investigation in simulated body fluid – SBF

The immersion study of PAA/HA/AuNPs was carried out in a solution of simulated body fluid SBF prepared according to Kokubo et al. [22]. The assessment of in vitro bioactivity was carried out by soaking the samples in a plastic container in 100 ml of SBF with pH = 7.34 and maintained at 37°C. The SBF solution had a composition and concentration similar to that of human plasma. The ion concentration of SBF (mmol) is given in table 1.

Table 1. The comparison of ion concentration and the pH value of SBF and human plasma [17]

	Concentation [mmol/dm^3]								**pH**
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}	
human plasma	142.0	5.0	1.5	3.5	103.0	27.0	1.0	0.5	7.2-7.4
SBF	142.0	5.0	1.5	3.5	147.8	4.2	1.0	0.5	7.4

Time of soaking was 14 days. After soaking, the specimens were removed from the fluid, washed with distilled water and dried to a constant weight.

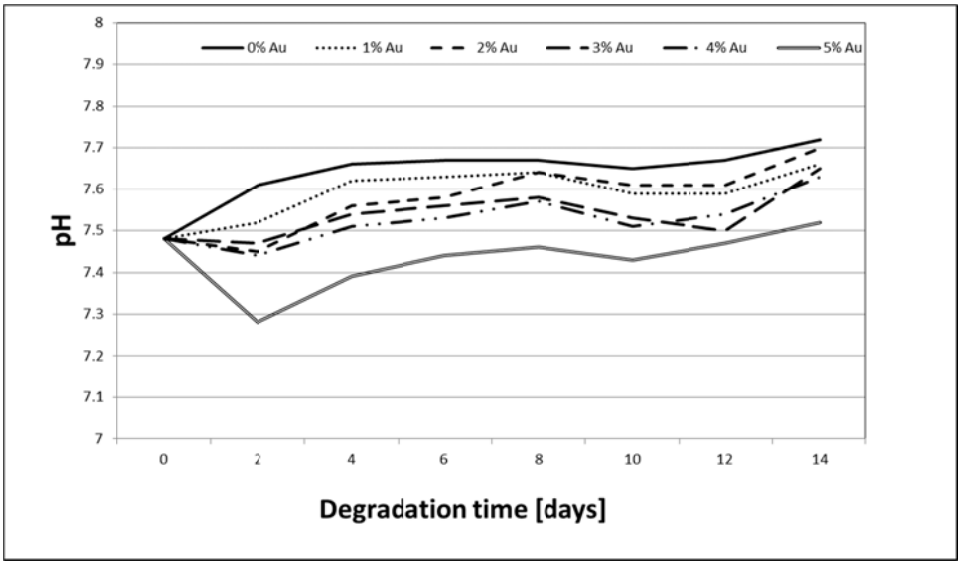

Fig. 3. The pH changes of simulated body fluid in the course of composites incubation

The chemical stability and biological activity were evaluated on the basis of pH changes recorded with time of immersion in physiological fluid - Simulated Body Fluid (SBF) - relevant to natural human environment (Fig. 3). The initial pH value of SBF was 7.34 at 25°C. Significant effect of gold nanoparticles content on the pH value of SBF was observed. For sample without nanoparticles increase of pH value was observed in the first period of immersion. On the fourth day of soaking the pH value stabilized at level of ~7.65 and was kept to the end of incubation. The growth of gold nanoparticles concentration caused decrease of pH value. The lowest pH value was observed for sample containing 5% AuNPs and amounted 7.28 after second day of incubation. Next, pH value gradually increased for all immersed samples.

Table 2. Results of gel permeation chromatography analyses (GPC) of Simulated Body Fluid

Sample	**Mw Weight average molecular weight**	**Mn Number average molecular weight**	**P Molecular weight distribution**
0% Au	241	249	0.97
1% Au	317	325	0.98
2% Au	286	294	0.97
3% Au	309	317	0.97
4% Au	301	309	0.97
5% Au	267	276	0.97

In samples with SBF as the degradation medium no polymeric or oligomeric compounds, except medium ingredients, were found. Therefore polymeric materials based on poly(acrylic acid), hydroxyapatite and gold nanoparticles are considered to be stable in Simulated Body Fluid. However, no significant differences correlated with gold concentration changes were visible.

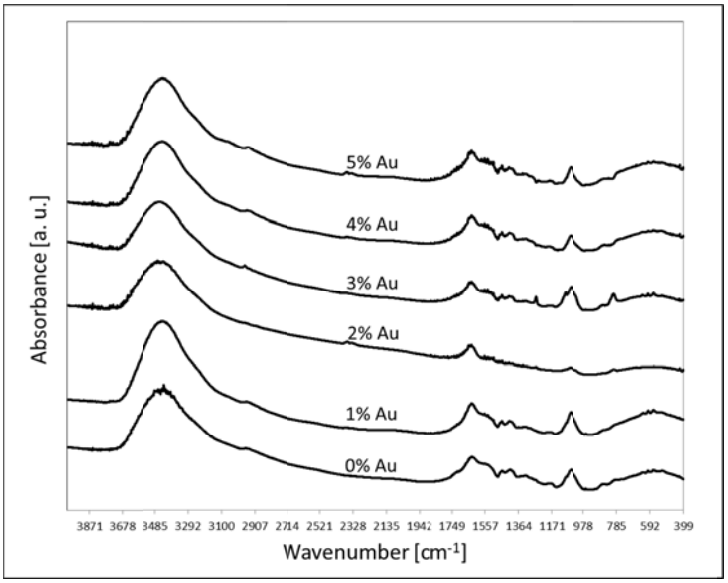

Fig. 4. FT-IR spectra of composites after incubation in SBF

FT-IR spectra of samples after incubation in simulated body fluid presents Figure 4. The obtained spectra are similar to each other. After comparison of spectra of samples before and after immersion distinct peak at 1040-1032 cm^{-1}, which corresponds to vibration of PO_4^{2-} groups, can be observed. The PO_4^{2-} group originated from hydroxyapatite incorporated in polymer matrix as well as hydroxyapatite growing on the samples surface. The hydroxyapatite agglomerates formed of SBF ions indicate that the composite samples exhibit biological activity.

3.4. Investigation in artificial saliva

In order to evaluation of application of hydrogel/hydroxyapatite/AuNPs composites in stomatology, incubation in artificial saliva was realized. The artificial saliva was prepared according to ISO standard [23] and its pH value was between 5.2 and 5.5. Composition of artificial saliva applied in in vitro tests presents Table 2.
Due to assessment of composites behavior in conditions of simulated oral cavity the pH value of artificial saliva was investigated during 14 days at 37°C.

Table 3. Composition of artificial saliva [23]

Component	Quantity [g/dm^3]
NaCl	0.400
KCl	0.400
$CaCl_2 \cdot H_2O$	0.795
$NaH_2PO_4 \cdot H_2O$	0.780
$Na_2S \cdot H_2O$	0.005
urea	1.000

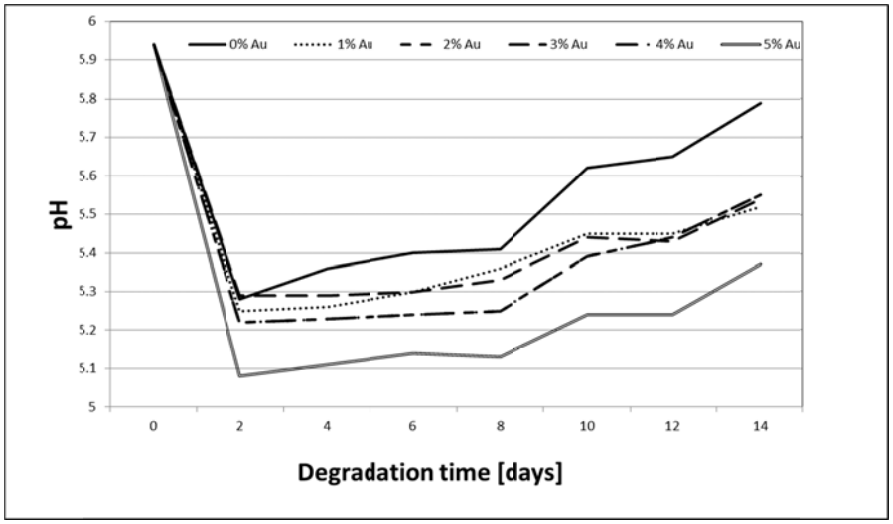

Fig. 5. The pH changes of artificial saliva in the course of composites incubation

Figure 5 presents changes of pH values of artificial saliva in function of incubation time. For all composite samples rapid decrease of pH value was noticed after second day of incubation, and amounted from 5.08 (5% Au) to 5.29 (0% Au). All samples were characterized with similar tendency in pH value changes. For sample contained the highest amount of AuNPs the lowest pH values was observed and next gradual and constant growth of pH was noticed. Samples containing 1% to 4% AuNPs caused similar growth of pH value of artificial saliva. However, for sample without gold nanoparticles the highest increase of pH value was noted.

Table 4. Results of gel permeation chromatography analyses (GPC) of artificial saliva.

Sample	**Mw Weight average molecular weight**	**Mn Number average molecular weight**	**P Molecular weight distribution**
0% Au	2 500	2 400	1.06
	29	33	0.86
1% Au	2 700	2 600	1.06
	31	35	0.89
2% Au	2 900	2 800	1.06
	30	34	0.89
3% Au	3 900	3 600	1.07
	30	33	0.89
4% Au	2 900	2 800	1.06
	30	34	0.89
5% Au	2 500	2 400	1.06
	26	29	0.89

In samples with artificial saliva used as the degradation medium only oligomeric fractions of polymer and low molecular solution ingredients were found. Therefore composite materials based on poly(acrylic acid), hydroxyapatite and gold nanoparticles are considered to be unstable in artificial saliva. The most interesting is sample with AuNPs concentration of 3%, where the degradation rate of polymer matrix was the lowest.

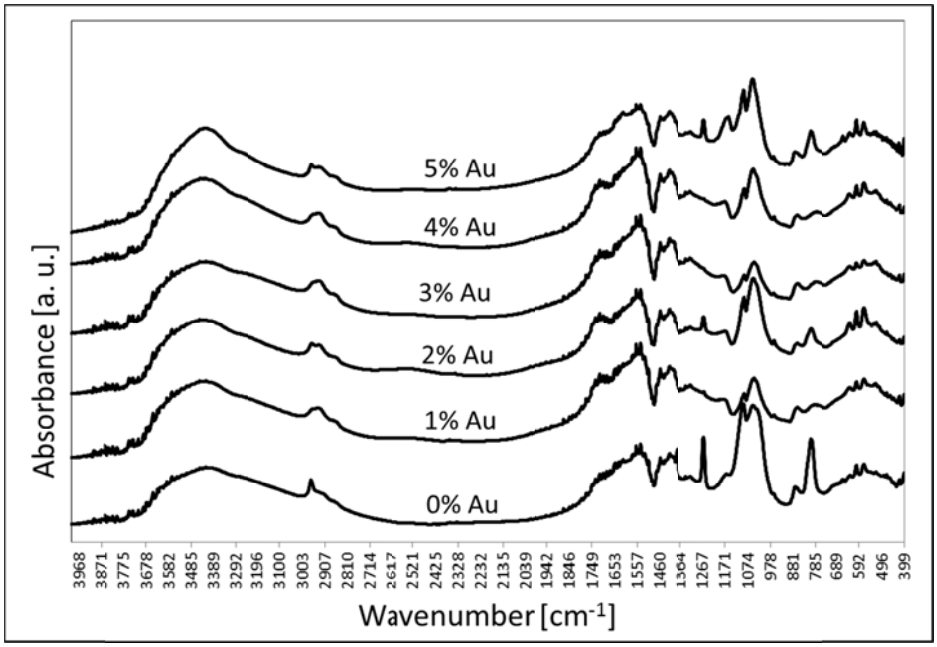

Fig. 6. FT-IR spectra of samples incubated in artificial saliva

The FT-IR analyses were realized for ceramic/polymer composites doped with AuNPs after immersion in artificial saliva at 37°C (Figure 6). The research results indicated that 14-days degradation period caused insignificant decomposition of polymer matrix. The FT-IR spectra after immersion in artificial saliva for all composite samples containing Au were of similar shape. However some peaks (at 797, 1078, 1099, 1260 cm^{-1}) were strengthened for sample without AuNPs. Infrared investigations confirmed that present of gold nanoparticles had considerably effect on composites structure.

4. Conclusion

In the environment of the living organism the behavior of polymer/ceramic composites with the addition of gold nanoparticles depends on chemical and phase composition, shape and the size of crystallites, presence of network defects, as well as biological conditions around the implant. The research on the influence of simulated body fluids on composite materials provides interesting information about stability in the environment of the living organism, but they are limited comparing with tests on cell lines or *in vivo* conditions. An undoubted advantage on incubative experiments in fluids simulating living organism is easiness of conducting them and lack of surgeries on tested animals.

The results of *in vitro* tests confirmed that it is possible to produce hydroxyapatite/polymer composites doped with gold nanoparticles for medical applications. Tests proved that content of gold nanoparticles in composites had influence on behaviour of HA/Polymer/AuNPs in artificial saliva and simulated body fluid. Measurements of pH value confirmed that composite materials characterises different *in vitro* behaviour.

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