https://doi.org/10.31925/farmacia.2023.1.11 **ORIGINAL ARTICLE**

PLGA-BISPHOSPHONATES CONJUGATED NANOPARTICLES: SYNTHESIS AND MORPHOLOGICAL CHARACTERIZATION

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Abstract

The aim of our study was to develop a more advanced method of using some already existing drugs by synthesizing a multicomponent biopolymeric nanocomposite material, which would function as a local drug release system. Thus, we started from simple polylactic-co-glycolic acid (PLGA) nanoparticles, synthesized by the double emulsion method. Then we functionalized the surface of the obtained material with polydopamine (PDA) to which we bound bisphosphonates (used in the current medical practice for the treatment of osteosarcoma). This resulted in a composite material with improved properties compared to constituent materials. The synthesized polymer biocomposites were characterized by SEM (morphological aspects) and Diffusion Light Scattering (DLS) (granulometric dimensions, zeta potential). The chosen synthesis method is simple, inexpensive, with easily adjustable parameters. The theoretical studies carried out regarding the bisphosphonate-polydopamine interaction showed a better stability for the risedronate interaction complex which agrees with the experimental data obtained for it.

Rezumat

Scopul studiului nostru a fost acela de a dezvolta o metoda mai avansată de utilizare a unor medicamente deja existente, prin sinteza unui material nanocompozit biopolimeric multicomponent, care să funcționeze ca sistem de eliberare locală a medicamentului. Astfel, am pornit de la nanoparticule de acid poli lactic-co-glicolic (PLGA), sintetizate prin metoda dublei emulsii, apoi am funcționalizat suprafața materialului obținut cu polidopamină (PDA) de care am legat bifosfonați (utilizați în practica medicală curentă în tratarea osteosarcomului) obținând un material compozit cu proprietăți îmbunătățite comparativ cu materialele constitutive. Biocompozitele polimerice sintetizate au fost caracterizate prin SEM (aspecte morfologice) si DLS (dimensiuni granulometrice, potențial zeta). Metoda de sinteză aleasă este simplă, necostisitoare, cu parametrii ușor ajustabili. Studiile teoretice efectuate asupra interacției bifosfonat-polidopamină au indicat o mai bună stabilitate a complexului de interacție pentru risedronat, ceea ce concordă cu datele experimentale obținute pentru acesta.

Keywords: PLGA, bisphosphonates, biopolymeric nanocomposites, molecular docking

Introduction

Composite materials were intensively studied in order to obtain drug release systems [1, 6]. About three decades ago, intensive research was carried out on the delivery of medicinal substances with the help of biodegradable polymers [2]. This research made it possible to use polymers as bioresorbable devices [16].
If a decade ago polymeric composites (PLA, PGA, PLGA, polyurethanes, polycaprolactones) or ceramic composites [20] (tricalcium phosphate, hydroxyapatite, bioactive glass) were used separately, in recent years research has focused on the synthesis of new materials that effectively combine the good mechanical properties with the biocompatibility determined by different components [7, 10].
The highest potency was observed at polylactic-co-glycolic acid (PLGA, a biodegradable polymer). PLGA has shown the property to deliver drugs [23] and serve as a support for tissue engineering [12].
According to the specialized literature, a great advantage of PLGA is that through degradation, it can release medicinal substances, without the need for surgical intervention [9]. Thus, through implantation, the drug is delivered in the appropriate dose, and the compounds resulting from degradation are non-toxic to the body. The required dose and the time at

which the drug is released are obtained controlling certain parameters considered relevant, such as the ratio of copolymers or the molecular weight of PLGA.
The ester bonds initiate degradation by hydrolysis, and the ratio of monomers used in the synthesis dictates the degradation time of PLGA. Thus, if glycolide is present in greater quantity, then the degradation time will be less than if lactide is predominant [29]. The exception, in this case, is the 50/50 monomer ratio, where the degradation is fast, in about 2 months.
The erosion of PLGA occurs heterogeneously, not homogeneously, being controlled by the degree of crystallinity of the polymer, molecular weight, hydrolysis, the pH and the temperature required in the glass transition, as well as by the lactic acid - glycolic acid molar ratio. Degradation occurs through the loss of molecular weight, leading to erosion and the total weight loss of the compound. With the loss of molecular weight, the transition temperature also decreases.
The flexibility of degradation made PLGA suitable for the production of medical devices such as nano-particles, implants or grafts.
To suit the required application, the properties of PLGA can be tailored to alter the drug release kinetics, nanostructure or encapsulation profile. If the dimensions are reduced, below 30 nm, PLGA becomes hydrophobic. Furthermore, it is detected by the endothelial reticulum and bound by phagocytes to be eliminated by the liver or spleen, all this before the delivery of the drug to the target.
To avoid this, the PLGA surface is functionalized by coating it with various substances such as PDA (polydopamine), which provide hydrophilic groups. This helps protect the structure from the detection of the endothelial reticulum. Apart from PDA, PEG, chitosan, poloxamines or poloxamer can also be used, which modify the hydrophobic properties of PLGA.
In order to facilitate the guidance of the vehicle to the target site of action and to increase the therapeutic efficiency, the PLGA surface is decorated with targeting ligands that have the property of binding to the receptors of the target cell [24].
The characterization of the binding behaviour of bisphosphonates to PDA plays an important role in elucidating the fundamental biochemical processes in which these compounds participate. Currently, the results reported in the specialized literature [8, 21] have indicated that the approach of ligand-biological receptor binding design (molecular docking studies and structure-activity/property correlation techniques) allows the exploration of probable binding conformations of biologically active compounds at the sites of the target protein. Furthermore, it provides useful information in understanding their structural and physico-chemical characteristics and, implicitly, in understanding their therapeutic effect. This helps to design new compounds with optimized activity.
The synthesis started from a copolymer of lactic and glycolic acid, PLGA, chosen for its special properties (biodegradability, non-toxicity, biocompatibility), accepted by the FDA, with the aim of a targeted local release of bisphosphonates. Those are beneficial in certain pathological processes such as osteosarcoma, and generally gave good results on various bone metastases. We started with the possibility of inducing a new way of administration, namely local administration.
In our paper, we used the combined approach between molecular docking and structure-energy correlation techniques for the binding of bisphosphonates to PDA. This last analysis was carried out with the help of geometric descriptors such as the molecular surface area and the volume of the studied molecules. These descriptors effectively characterize the shape of the molecule, the shape that plays an essential role in the ligand – biological receptor interaction [3, 4]. Therefore, the actual interaction of the two is preceded by the steric accommodation of the ligand in the active site of the receptor, being as better as the shape of the molecule allows this.

Materials and Methods

All reagents and chemicals are of analytical grade. Both PLGA (65:35, Mw 40000 - 75000) and dopamine chloride were purchased from Sigma-Aldrich. DCM, PVA 8-88 and Water LiCroSolv were acquired from by Merck, Darmstadt, Germany; Biphosponates such as alendronate monosodium trihydrate, disodium pamidronate, risedronate sodium were purchased from Merck, whereas zolendronate disodium salt was procured from ABCAM. Tris base buffer was achieved from Roth.
Synthesis. The synthesis is based on methods already described by us in specialized literature with modifications [11].
PLGA nanoparticles. Aqueous phase A_1 was represented by a 5 mL aqueous solution with 0.5% polyvinyl alcohol (PVA). The oil phase was prepared by dissolving 100 mg of PLGA in dichloromethane (DCM). The two phases were mixed at 45000 rpm in a vortex (Heidolph Silent Crusher) to obtain the A_1/O primary emulsion.
Aqueous phase A_2 was represented by an aqueous solution (95 mL) of 0.5% polyvinyl alcohol which acted as an emulsifier (w/w). The primary emulsion was added to the second aqueous phase and stirred at 1000 rpm for 3 hours to evaporate the DCM. The final suspension was centrifuged and then subjected to a lyophilization process (Labconco FreeZone 18 Liter Console Freeze Dry System), left overnight at -45ºC, then kept for 10 hours at 0,014 mbar and -9°C.
Secondary drying stage 2: at 0,0014 mbar and 20°C for 10 hours. PLGA nanoparticles were kept at 5°C for later use.

Surface functionalization. The obtained material (PLGA nanoparticles) was added to 5 mL of aqueous solution brought to pH = 8.5 (0.01 M Tris base buffer) containing 10 mg of dopamine and stirred for 3 h at room temperature to coat the surface of PLGA with PDA. 20 mg bisphosphonate (pamidronate/risedronate/ alendronate/zoledronate) dissolved in 50 mL water and 100 mg PLGA-PDA particles were added under continuous stirring for 5 h. The final suspension of PDA-biphosphonate-conjugated nanoparticles was centrifuged at 11000 rpm and washed.

Molecular docking and geometrical descriptors. The chemical structure of bisphosphonates was constructed using HyperChem Release 8.0 Professional software (Hypercube Inc.) [14]. The conformational analysis of these compounds was performed by the semi-empirical PM3 calculation method.

The resulting geometry was transferred to the Hex 8.0 program [22], which performed the docking calculations and provided the ligand (bisphosphonate)-receptor (polydopamine) interaction energies. The structure of PDA could not be downloaded from the PDB database [15], because its structure is currently not known precisely [17, 30]. The structure was achieved by repeated docking of the tetrameric subunits leading to layered aggregates as predicted by the recently reported studies [25].

The geometrical descriptors were calculated using HyperChem Release version 8.0 Professional software (Hypercube Inc.).

Morphological characterization. SEM image acquisition was performed with a high-resolution scanning electron microscope, FEI Inspect F50, at 30 KeV voltage and at various magnifications.

Granulometric size. For particle size analysis, a Zeta Potential Analyzer (Brookhaven) was used. Accuracy ± 2%; repeatability: ± 2%; laser: 35 mW solid state, red light (660 nm wavelength); working temperature 25ºC; diffraction angle 90º.

The samples were diluted in water and sonicated for 5 minutes. The cuvette was filled with dispersant liquid specific to the sample to be analysed (1.5 mL). The sample to be analysed was subjected to the ultrasound process to break any agglomerates and the porous material was eligible for analysis. For a correct analysis, a minimum of 5 measurements of 2 minutes each were performed.

Results and Discussion

Synthesis

Our composite material is part of the newest generation of composite materials with local drug release (biopolymeric nanoparticles). It represents a real interest for the entrepreneurial sector because it offers a series of advantages compared to the conventional systemic administration (oral, parenteral) [26] such as: avoids drug concentration fluctuations, overdose, systemic toxicity, hepatic and renal side effects. Furthermore, it provides local release at bone level where blood flow does not reach in sufficient quantity to ensure an adequate drug concentration if oral administration is used.

In our case, PDA [13] was used in order to bind bisphosphonates (risedronate, zolendronate, alendronate, pamidronate) to our system. Both PDA and bisphosphonates have important roles. PDA has the advantage of being water soluble, solubility required according to the chosen synthesis method, while bisphosphonates are very important, due to the very high affinity for calcium ions and implicitly for the bone system (hydroxyapatite being the main inorganic component of the bone). They show a regenerative action of the bone system through osteoblastic stimulation.

Molecular docking and geometrical descriptors

The best docking results were considered to be the conformations with the lowest docking energy which are listed in Table I. PDA was obtained by repeated docking of eight tetrameric subunits, the latter being combined in two ways, by chemically linking the same mer - dihydroxyindole (simple oligomer) and, respectively, by covalent binding of four different mers in equimolecular ratio (dopamine, dihydroxyindole, leukodopaminechrome and dopaminechrome; mixed oligomer) in the most reactive positions 2, 4 and 7 (Figure 1).

Table I

Bisphosphonate-PDA interaction energies (kJ/mol)

Compound	***8 tetramers*** (simple oligomer)	***8 tetramers*** (mixed oligomer)
Alendronate	-153.73	-156.21
Pamidronate	-156.23	-153.46
Risedronate	-186.00	-184.92
Zolendronate	-185.25	-179.43

The results (Table I) show that the most stable bisphosphonate-PDA complex (having the lowest binding energy, -186.00 kJ/mol and -184.92 kJ/mol, respectively) is the one involving riserdronate, followed by zolendronate, alendronate and pamidronate. This may impose a slow degradation kinetics for the risedronate-PDA complex, ensuring the delayed release of the bisphosphonate at the bioactive target and thus a better therapeutic action of it compared to the others.

The results also show approximately equal values between the binding energies of bisphosphonates with the two forms of oligomers present in PDA. This marks the fact that, regardless of the structure of PDA, risedronate forms the most stable complex with PDA.

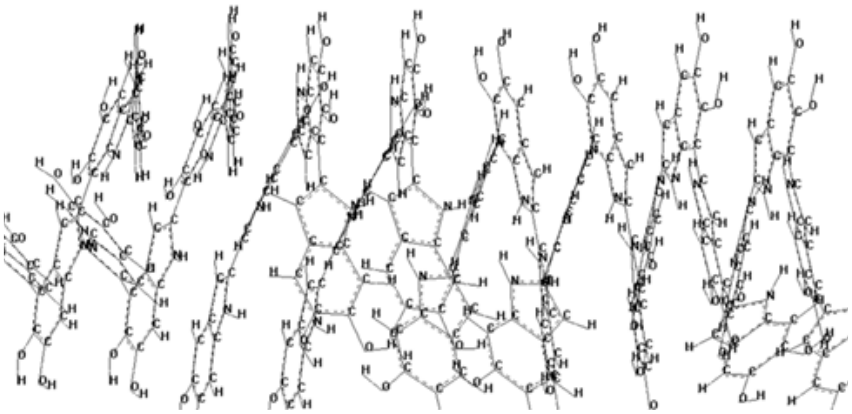


Figure 1.
The layered aggregate structures formed by eight of the most stable

This result is also supported by the idea of a better accommodation of risedronate in the active site of PDA, due to a larger geometry and possibly a more suitable value for the geometry of the active area of PDA receptor. Table II shows the values of the geometric descriptors calculated for the studied compounds.

Table II
Geometric descriptors calculated with Hyperchem software

Compound	$E_{binding}$, kJ/mol	*SA*, Å^2	*V*, Å^3
Alendronate	-156.21	374.33	629.92
Pamidronate	-153.46	341.50	571.92
Risedronate	-184.92	399.41	678.63
Zolendronate	-179.43	381.57	646.08

As can be seen in Table II, the decreasing order of the stability of the bisphosphonate-PDA complex (which corresponds to the increasing variation of the $E_{binding}$ interaction energy) is the same as that of molecular surface or volume for the studied compounds, *i.e.* risedronate > zolendronate > alendronate > pamidronate.

From here on, the results presented will be for the PLGA-Riserdronate (PLGA-Ris) complex, specifying that regardless of the nature of the bisphosphonate used; the morphological aspects, dimensions and Zeta potential did not show substantial changes.

Morphological Aspects

In the images related to each sample (those for the PLGA-Ris composite shown in Figure 2), taken at low magnifications of 500x, 600x, the porous spherical structure characteristic of all materials with PLGA obtained by the double emulsification method can be observed.

The conjugation of PLGA with bisphosphonates slightly changes the morphology of the material. Moreover, the synthesized composite materials still have heterogeneous characteristics, due to the multiple components of the samples. It is assumed that bisphosphonates bind to PDA *via* peptide bonds on the spherical polymeric surface [19].

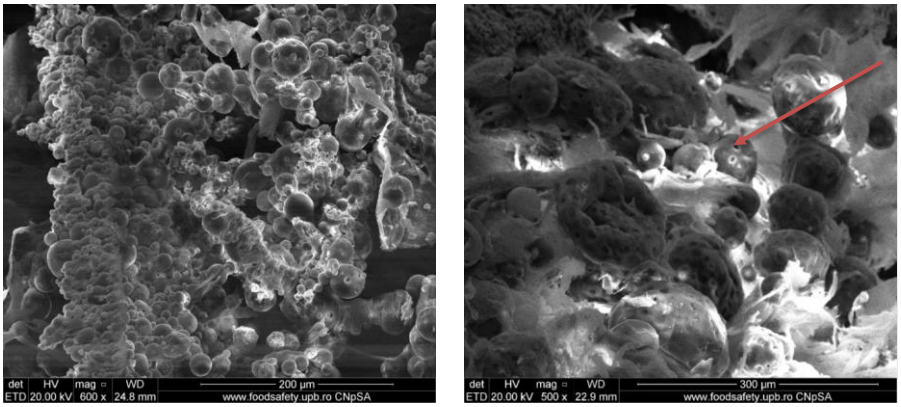


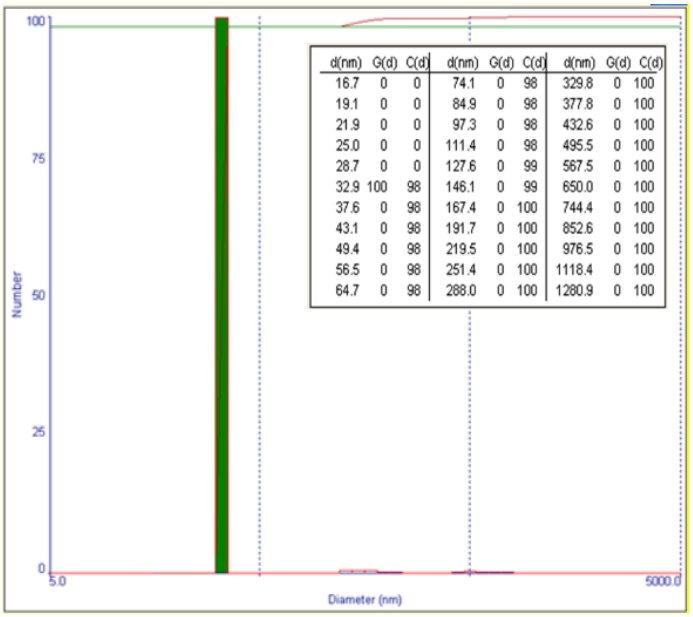
Figure 2.
PLGA-Riserdronate morphology,at different magnitudes

There is a link between the synthesis method (double emulsion *versus* simple emulsion), the stirring speed, the volume of the secondary aqueous phase (which implicitly leads to a different volatilization of DCM) and a different diffusion of the initial aqueous phase [28]. Thus, a high stirring speed with a secondary aqueous phase smaller in volume leads to a more accentuated volatilization of the organic solvent. As it volatilizes, PLGA begins to precipitate and the water from the initial aqueous phase comes out through diffusion, forming the pores observed by SEM [5]. The double emulsion method is the one that leads to formation of pores on the surface of the material, unlike the simple oil-water emulsion. This is determined by the internal aqueous phase.

DLS analysis

Diffusion Light Scattering (DLS) was able to evaluate the particle size distribution (hydrodynamic diameter), and the scattering effect of electromagnetic radiation by particles depending on the ratio between the particle size and the wavelength of the electromagnetic radiation [18]. The number distribution shows that the single, unconjugated PLGA particles have a size of 32 nm (Figure 3). The data resulted from the volume distribution exhibit larger particles of [127 - 220] nm and [432 - 745] nm, but they are not visible in the case of numerical distribution due to their insignificant number.

A



B

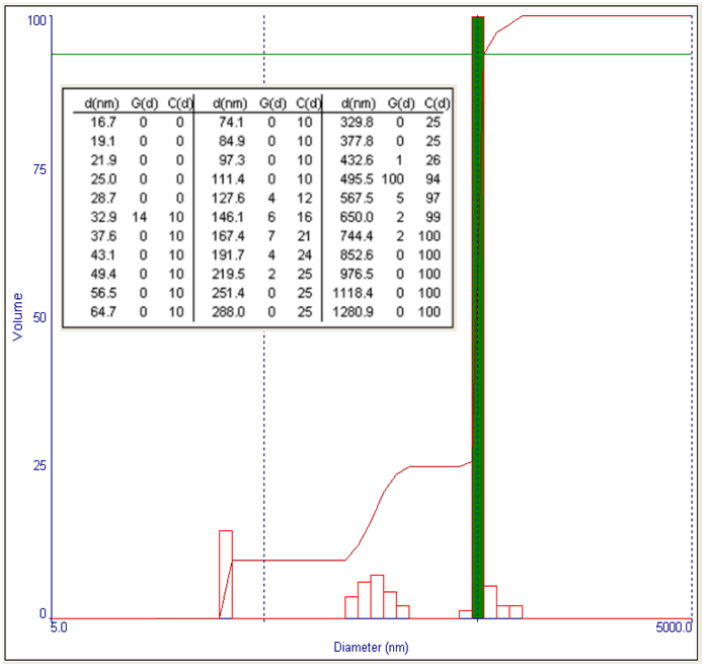
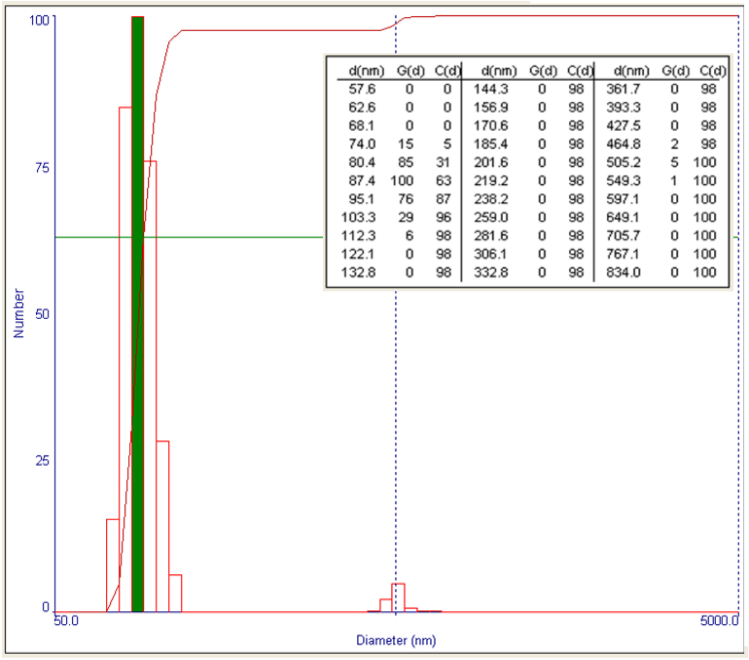


Figure 3.

A) Numerical and B) volumetric distribution of centrifuged PLGA particles

A



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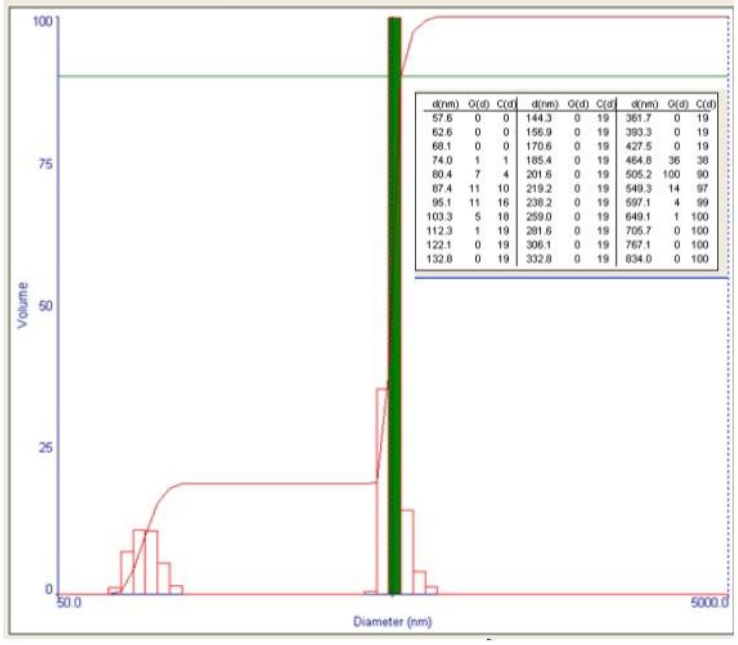

Figure 4.
A) numerical and B) volumetric distribution of PLGA-Ris

From the analysis of the data regarding the granulometric analysis (by volume and by number) of PLGA-Ris (Figure 4), the presence of two granulometric intervals was found, namely: [74 - 113] nm respectively [464 - 650] nm. If we strictly analyse the numerical distribution, it can be seen that the largest number of particles have a size of around 87 nm, and the particles that form the majority in the case of volume distribution (the largest volume being around the size of 505 nm) are in very small number.

The negative values of the zeta potential for composite materials are mentioned in specialized literature for materials having a very good stability. Even by conjugation with PDA and bisphosphonates, the material retained its negative zeta potential (unconjugated PLGA -42.09 mV and for PLGA-Ris -32.40 mV) (Figures 5 and 6).

Our results are supported by a series of studies showing that a material with an electronegative surface charge is more accessible for osteoblast attachment and proliferation because negatively charged species (mesenchymal cells and osteoblasts) are attracted when in intimate contact [6].

The bisphosphonate-PDA interaction studies carried out by molecular docking indicated an increased stability for riserdronate and zolendronate, in accordance with the data from the literature that show their increased potency compared to other bisphosphonates [27].

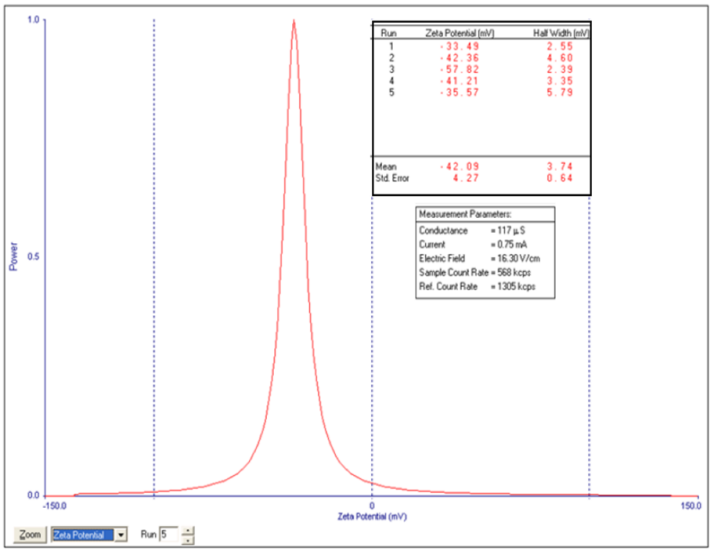

Figure 5.
Zeta potential of unconjugated material, PLGA

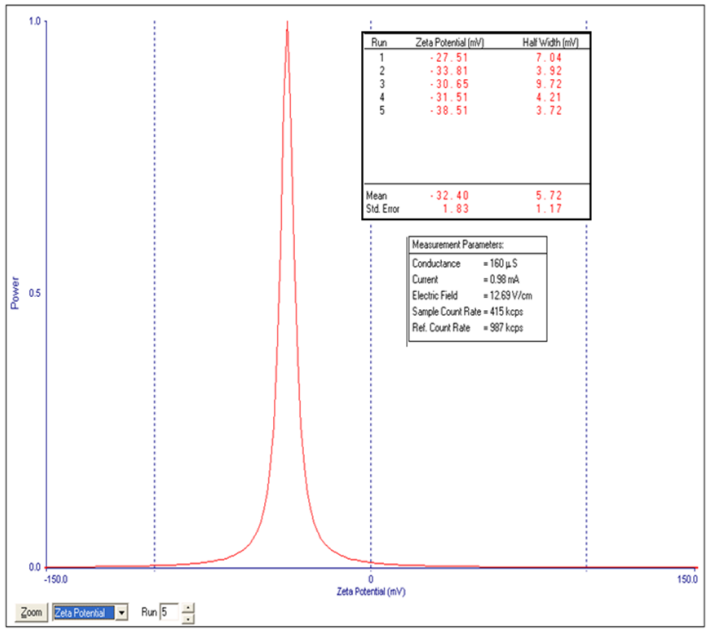

Figure 6.
Zeta potential of PLGA-risedronate conjugated composite material (PLGA-Ris)

Conclusions

The chosen synthesis method is used for the composite material due to its simplicity and the extremely low costs involved. The functionalization of the PLGA surface with PDA to which we linked bisphosphonates offers advantages for the subsequent use of the composite material in bone metastases, bisphosphonates stimulating osteoblastic proliferation.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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