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1 **Fabrication of 3D printed antimicrobial polycaprolactone scaffolds for tissue**
2 **engineering applications**

3

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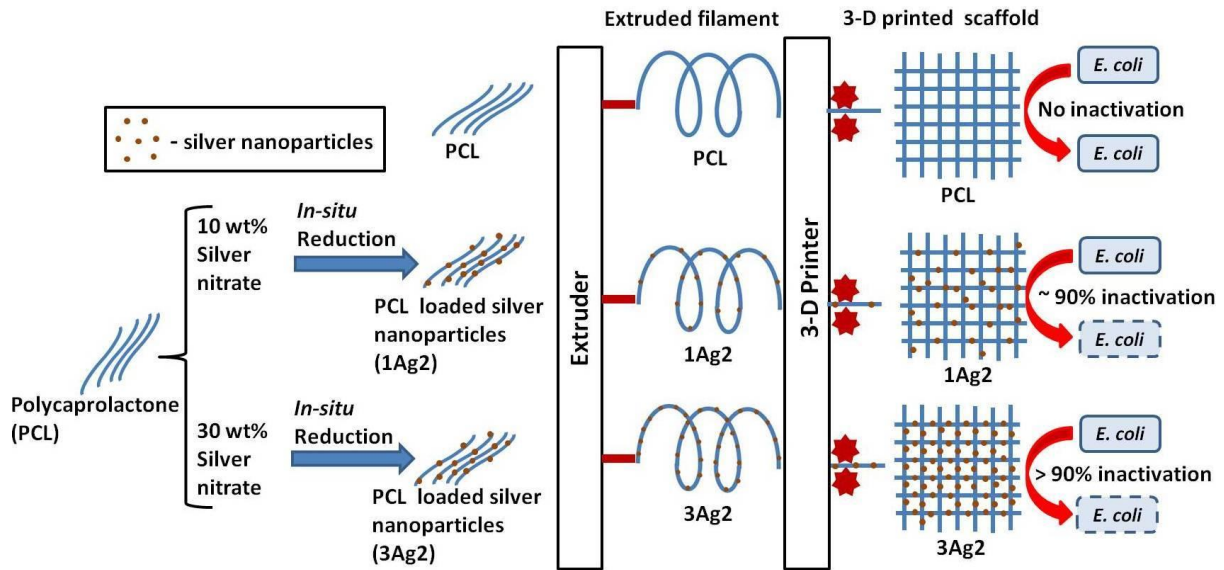
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1 **GRAPHICAL ABSTRACT**



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4 **ABSTRACT**

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Synthetic polymers are widely employed for bone tissue engineering due to their tunable physical properties and biocompatibility. Inherently, most of these polymers display poor antimicrobial properties. Infection at the site of implantation is a major cause for failure or delay in bone healing process and the development of antimicrobial polymers is highly desired. In this study, silver nanoparticles (AgNps) were synthesized in polycaprolactone (PCL) solution by in-situ reduction which was further extruded into PCL/AgNp filaments. Customized 3D structures were then fabricated using the PCL/AgNp filaments through 3D printing technique. As demonstrated by scanning electron microscopy, the 3D printed scaffolds exhibited interconnected porous structures which are crucial for cell infiltration. Furthermore, X-ray photoelectron spectroscopy analysis revealed the reduction of silver ion. Transmission electron microscopy along with energy-dispersive X-ray spectroscopy analysis revealed the formation of silver nanoparticles throughout the PCL matrix. *In vitro* enzymatic degradation studies showed that the PCL/AgNps scaffolds displayed 80% degradation in 20 days. The scaffolds were cyto-compatible, as assessed by using MG63 cells and their antibacterial activity was demonstrated on *Escherichia coli*. Due to their interconnected porous structure, mechanical and antibacterial properties, these cyto-compatible multifunctional 3D printed PCL/AgNps scaffolds are highly suitable for bone tissue engineering.

1 **Keywords:**

2 polycaprolactone, silver nanoparticles, nanocomposites, cytocompatibility, antimicrobial,
3 multifunctional properties

4
5 **Highlights:**

6 We report the development of antimicrobial polycaprolactone scaffold by 3D printing

7 The silver nanoparticles (AgNps) were obtained by in-situ reduction in PCL solution

8 AgNps reinforcement improved the stiffness of the 3D printed scaffolds

9 3D printed scaffold exhibited multifunctional property

10 Fabricated 3D scaffold showed cytocompatibility and antimicrobial properties

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13 **INTRODUCTION**

14 Fused deposition modelling (FDM) is one of the simple and cost effective techniques
15 employed in 3D printing of polymers for bone tissue engineering¹⁻². Various thermoplastic
16 polymers such as poly-ε-capro-lactone (PCL)³, poly(L-lactic acid) (PLLA)⁴ and poly-vinyl-
17 alcohol (PVA)⁵, have been used in 3D printing. Among them, PCL was widely used for the
18 fabrication of bone scaffolds because of its FDA (Food and Drug Administration) approval,
19 biocompatibility and biodegradation properties⁶. The low melting point (60°C) of PCL is
20 advantageous for 3D printing⁷. In addition, PCL exhibits good mechanical properties with
21 high flexibility and great elongation at break⁸, interesting for the preparation of scaffolds for
22 craniofacial bone repair and cranial defects⁹. However, optimization of 3D printed PCL
23 scaffolds by including other multifunctional properties such as antimicrobial activity and
24 mechanical performance, without compromising their biocompatibility, is crucial to employ
25 the scaffold for biomedical application.

26 About 2 to 5% of orthopaedic implants are complicated by infection¹⁰ and this can
27 raise up to 30% in the case of open fractures¹¹. Implant related infections are associated with
28 significant morbidity and sometimes death. These complications lead to implant removal,
29 surgical debridement of infected tissue and long-term antibiotic administration¹²⁻¹³. Loading
30 of antibiotics or metallic component is an issue to impart antibacterial property to the
31 scaffolds. For instance, Visscher *et al.* developed 3D printed PCL scaffolds containing
32 macro- and micro-pores (obtained using porogen) and coated with antibiotic (@Cefazoline).
33 These scaffolds were active against *S. aureus*¹⁴. This design is very potential, however the

1 alarming levels of antibiotic resistance bacteria implies the necessity for developing potential
2 alternate to this approach.

3 Silver, copper and zinc as metallic components are shown to exhibit antibacterial properties¹⁵.
4 Among them, silver has been widely used in research and clinics as a broad spectrum
5 antibacterial agent and apart from antibacterial application, silver nanoparticles-polymer
6 composite has been used as catalytic agent¹⁶, super capacitors¹⁷, electromagnetic interference
7 (EMI) shielding¹⁸, fuel cells¹⁹ etc., Mechanism of the antibacterial activity of silver
8 nanoparticles (AgNps) is unclear. However, a recent investigation by Qing *et al.* provides
9 possible explanations: (i) Anchoring of AgNps on bacterial membrane leads to membrane
10 rupture and cellular content leakage that result in the bacterial cell death, (ii) Small size
11 nanoparticles infiltrate the membrane and interact with the biomolecules which result in the
12 dysfunction of bacterial cell, and (iii) AgNps are likely to catalyze O₂ reduction into reactive
13 oxygen species (ROS) whose accumulation leads to down regulation of antioxidant enzyme
14 expression, DNA damages and apoptosis²⁰.

15 Silver ions and its metallic nanoparticles can be directly incorporated into inorganic
16 materials or mixed with synthetic and biopolymeric materials for the fabrication of
17 antibacterial 3D printed structures^{21 22}. For example, Muwaffak *et al.* developed 3D printed
18 antimicrobial wound dressing material by incorporating ionic silver, copper and zinc oxide
19 onto the PCL polymer. Silver and copper based PCL material displayed better bactericidal
20 property than the zinc based material. However, this study is very limited and the influence of
21 ionic silver on biocompatibility was not analyzed.²³ AgNps were mixed with other synthetic
22 polymers such as polyetheretherketone (PEEK) and 3D printed using FDM technique. The
23 obtained scaffolds showed good antimicrobial properties²⁴. Silver incorporation in tricalcium
24 phosphate (as inorganic matrix) based 3D printed scaffolds facilitated differentiation of
25 mesenchymal stem cells into osteoblasts²⁵. PCL scaffolds were also synthesized with
26 impregnated or surface coated silver nanoparticles in order to increase antibacterial activity²⁶⁻
27 ²⁸. The scaffolds fabricated through solvent casting and electrospinning technique display
28 poor control over the architecture and the uncontrollable pore size in comparison to the 3D
29 printing approach.

30
31 In this work, silver ions were *in situ* reduced into silver nanoparticles and
32 encapsulated into the PCL matrix. The PCL composite (PCL/AgNps) was thermally extruded
33 into PCL/AgNps filaments used for the fabrication of customized scaffolds by the FDM
34 technique. After validation of *in situ* reduction, the enzymatic degradation of the scaffolds

1 was elaborately analyzed. The antibacterial activity of the PCL and PCL/AgNps scaffolds
2 was analyzed. Furthermore, scaffold cytocompatibility was evaluated using hFOB cells in
3 order to assess their potential use for bone tissue engineering applications.

4 5 **Materials and Methods**

6 **Materials**

7 Polycaprolactone (PCL, Mw~ 50000 Da) pellets were obtained from polymorph
8 (UK). N,N-Dimethylformamide (DMF; 99.8%; CAS number 68-12-2; Sigma),
9 Tetrahydrofuran (THF; 99%; CAS number 109-99-9; Sigma), silver nitrate (AgNO₃; CAS
10 number 7761-88-8, Sigma) , Lipase cepacia (40.7 units/mg, CAS number 9001-62-1; Sigma),
11 MEM α (Minimum Essential Medium α) (A10490-01; Gibco), MTT (3-(4,5-Dimethylthiazol-
12 2-yl)-2,5-Diphenyltetrazolium Bromide) (98%; CAS number 298- 93 -1), fetal bovine serum
13 (FBS) (CVFSVF00-01; Eurobio), 1% (V/V), penicillin/streptomycin (15140-122; Gibco),
14 0.05% Trypsin-EDTA (25300-054; Gibco), Dimethyl sulfoxide (DMSO) (23486.297; BDH
15 Prolab), ethanol (96% vol; CAS number 64-17-5), phosphate buffered saline (PBS, P44717;
16 Sigma), Lysogeny broth (LB) Miller culture medium (L3522; Sigma), microbiologic agar
17 (A1296-1KG; Sigma), Bacteriopeptone (211677-500g; BD Biosciences), Yeast extract
18 (9112001; Biokar diagnostics), Sodium Chloride (NaCl; 99%; CAS number 7647-14-5;
19 Sigma), Potassium dihydrogen phosphate (KH₂PO₄; CAS number 7778-77-0), di-Sodium
20 hydrogen phosphate dodecahydrate (Na₂HPO₄.12H₂O ; CAS number: 10039-32-4 ; Sigma)
21 and agar (CAS number 9002-18-0; Sigma) were obtained and used without further
22 purification. hFOB osteoblastic cell line obtained from ATCC was used for cytocompatibility
23 analysis. Non-pathogenic Gram-negative *Escherichia coli* bacterium (K12 DSM 423, from
24 DSMZ, Germany) was used for the antibacterial study.

25 26 **Preparation of PCL/AgNps composite and extrusion of composite filaments**

27 PCL solution (10% w/v) was prepared by dissolving it in solvent mixture (7:3 volume
28 ratio of THF and DMF respectively) at 90°C. Silver nitrate with 2 different percentages (1%
29 and 3% w/v) was added into the PCL solution and the temperature of the solution was
30 maintained at 90°C. Resultant solution was refluxed for 3 hours to facilitate the reduction of
31 silver nitrate into silver nanoparticles. Brown polymer suspension was obtained which are
32 cooled and precipitated using 300 mL of ethanol and dried at room temperature. PCL/AgNp
33 composites prepared using 1% and 3% silver nitrate are denoted as C1Ag2 and C3Ag2. PCL,
34 C1Ag2 and C3Ag2 were extruded into filament by Noztek pro extruder at 120 °C.

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Fabrication of 3D printed PCL/AgNp scaffold

PCL/AgNp scaffolds were fabricated using FDM technique. Scaffolds structure was customized using DesignSpark Mechanical® 2.0 software. The extruded filament was cast into 3D printed matrix by using FDM based 3D printer (PRUSA i3) and the settings are reported in the Table 1. Furthermore the scaffold made from the filaments of PCL, C1Ag2 and C3Ag2 are denoted as PCL, 1Ag2 and 3Ag2.

Table 1 Printer settings employed for printing PCL and PCL/AgNp scaffolds

Nozzle Diameter	0.4 mm
Extrusion width	0.45 mm
Layer height	0.05 mm
Fill density	25%
Fill pattern	Cubic
Extruder temperature	140°C
Bed Temperature	25°C
Speed of print moves	20-30 mm/s

Physical and chemical characterizations of the polymer and scaffolds

Surface morphology of the scaffold was observed using HITACHI S4800 scanning electron microscopy system and the samples were sputter-coated for 30s using platinum/palladium for SEM analysis. Here, Image J software was employed to calculate the mean filament diameter by taking average at 20 points and the pore size by taking average of 8 pores, which is denoted as mean \pm standard deviation. The Fourier transform infrared (FTIR) spectrum of the scaffold was recorded in the frequency range of 4000 – 600 cm^{-1} by NEXUS instrument in an attenuated total reflection (ATR) mode. X-Ray diffraction (XRD) analysis was performed by PANalytica Xpert powder XRD system using Cu $K\alpha$ radiation, 2θ range of 10–80°. X-ray photoelectron spectroscopy (XPS) analysis was performed using KRATOS- AXIS Ultra DLD model having monochromatic Al-K alpha source of 1486.6 eV spectral resolution FWHM 0.45 eV. *In situ* synthesis of silver nanoparticles in the PCL polymer was characterized using TEM (JEOL ARM 200F) combined with an EDX Analyzer.

1 For TEM analysis, polymer samples were cut into 90 nm thick slices after fixing it in Devcon
2 5@Minute Epoxy using ultra microtome and mounted over the Cu grid. Mechanical
3 properties of the PCL composites were assessed using a traction machine (MTS 1/ME)
4 equipped with a 5 kN force sensor. PCL composites were 3D printed as standard dog bones
5 shapes to measure the mechanical properties and the analysis was performed in the strain rate
6 of 0.01 mm s⁻¹. Furthermore, Young's modulus and Poisson's ratio were calculated on
7 minimum three assays as demonstrated elsewhere²⁹⁻³⁰.

9 **Enzymatic degradation of the PCL and PCL/AgNp scaffolds**

10 *In vitro* degradation of the scaffolds was carried out using lipase enzyme at
11 concentration of 10 units/mL. The lipase solution was prepared from 0.01 M phosphate-
12 buffered saline (PBS) at pH 7.4. The samples were weighed and placed in a vial containing
13 the 1 ml lipase solution and incubated at 37°C. Every 3 days, the enzyme solution was
14 replenished with fresh lipase solution. After definite time, the samples were removed from
15 the enzyme solution, washed, dried at 37°C and weighed. The percentage of degradation of
16 the scaffold was calculated by measuring the ratio of the final weight of the sample to that of
17 the initial weight (taken in the range of 9.0-15.0 mg) of the sample that is after and before the
18 degradation.

20 **Cell viability assay**

21 hFOB osteoblastic cells were cultured using DMEM/F12 (Dulbecco's Modified Eagle
22 Medium α) (Gibco 10565018) conditioned media supplemented with (i) 10 % (V/V) fetal
23 bovine serum (FBS) (Eurobio CVFVSF00-01). Cells were cultured at 37°C in 5% CO₂ in a
24 10 cm diameter petri dish and trypsinized using 0.05% Trypsin-EDTA (Gibco 25300-054).
25 After sterilization with 70% (w/v) ethanol for 30 mins, the printed scaffolds were dried at
26 room temperature and then placed in contact with hFIB cells for 8 days. Cell viability was
27 analyzed using MTT assay carried out by incubating 100 μ L of 0.5 mg/mL of MTT solution
28 on the cells for 3h. Purple coloured formazan crystals were dissolved using 40 μ L of DMSO
29 (BDH Prolab 23486.297) and the absorbance was recorded at 560 nm using Multiskan plate
30 reader (thermos, USA).

32 **Antibacterial activity**

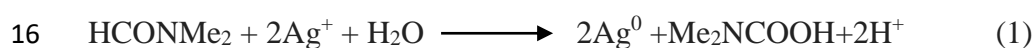
33 Antibacterial activity of the 3D PCL/AgNp scaffold was characterized by soft agar
34 and liquid tests to study bacterial growth inhibition and bactericidal action respectively (the

1 detailed protocols are given in the supportive information S1). The samples used for the
2 antibacterial activity trials were sterilized by immersion in a water/ethanol (3:7 v/v) solution
3 for 1 h and washed afterwards for several times with sterile deionized water to remove the
4 ethanol traces in the sample. Later the washed samples were sterilely dried at 30°C overnight
5 and used for the antibacterial activity tests. Bacterial growth inhibition tests were carried out
6 by putting the samples in contact with a nutritive soft agar inoculated with bacteria.
7 Bactericidal action of the material was characterized by incubating the material in a
8 suspension of resting bacteria (i.e., un-growing bacteria).

9

10 **RESULTS AND DISCUSSION**

11 PCL/AgNps composite was synthesized through in situ reduction –mechanism
12 (equation 1 and 2) as described by Santos et al.³¹. Obtained PCL/AgNp was utilized for the
13 fabrication of customized 3D printed structure (Figure 1). Physicochemical properties,
14 biocompatibility as well as antimicrobial properties of the PCL and PCL/AgNps scaffolds
15 were analyzed and discussed.



17 Santos et al.³¹. described that the carbamic acid undergoes decomposition at high temperature
18 followed by evaporation.



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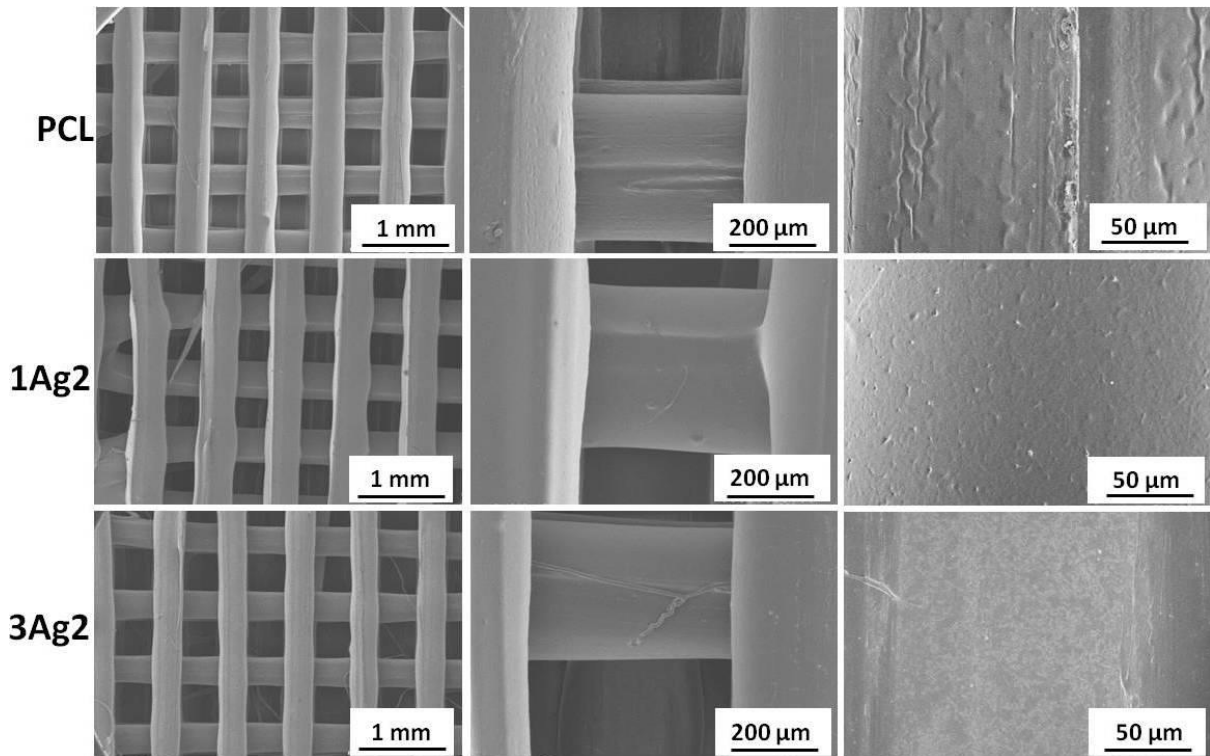
22 Figure 1. Photograph of the 3D printed scaffolds (scale bar denotes 1cm)

23

24 **Physicochemical properties of the scaffolds**

25 Scanning electron microscopy SEM images of the 3D printed PCL and PCL/AgNps scaffolds
26 are shown in the Figure 2. SEM micrographs revealed that the PCL and PCL/AgNps 3D
27 scaffolds matrix displayed uniform porous structures with open and interconnected pores.
28 Inter filament spacing resulting from the fusion of multiple layers of polymer filaments are
29 denoted as pores as mentioned in the literature^{21, 32}. 3D structure and pore size of the

1 scaffolds plays a crucial role in cell infiltration. Murphy *et al.* demonstrated that the scaffolds
 2 having 80 to 190 μm pore size display cell aggregation and poor cells infiltration. Scaffolds
 3 of 300 μm pores size possess improved cells infiltration and the cells were observed at the
 4 center of the scaffolds without cell aggregation. These results prove that the pores sizes above
 5 300 μm is crucial in the scaffolds design³³. Scaffolds obtained in our study displayed pore
 6 size larger than 300 μm (Table 2), this dimension being suitable for osteoblast cell
 7 infiltration. The diameter of 3D printed filaments is also shown in Table 2. SEM micrographs
 8 of 1Ag2 and 3Ag2 revealed homogeneous filament diameters which is crucial in order to
 9 obtain scaffolds with uniform architectures. High magnification SEM micrograph reveals
 10 wrinkly surface morphology for PCL and almost smooth and uniform surface for 1Ag2 and
 11 3Ag2 (Figure 1). Here, AgNps act as the inorganic filler leading to the increased stiffness
 12 which is evident from the increased Young's modulus.



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 14 **Figure 2.** SEM analysis of 3D printed scaffolds

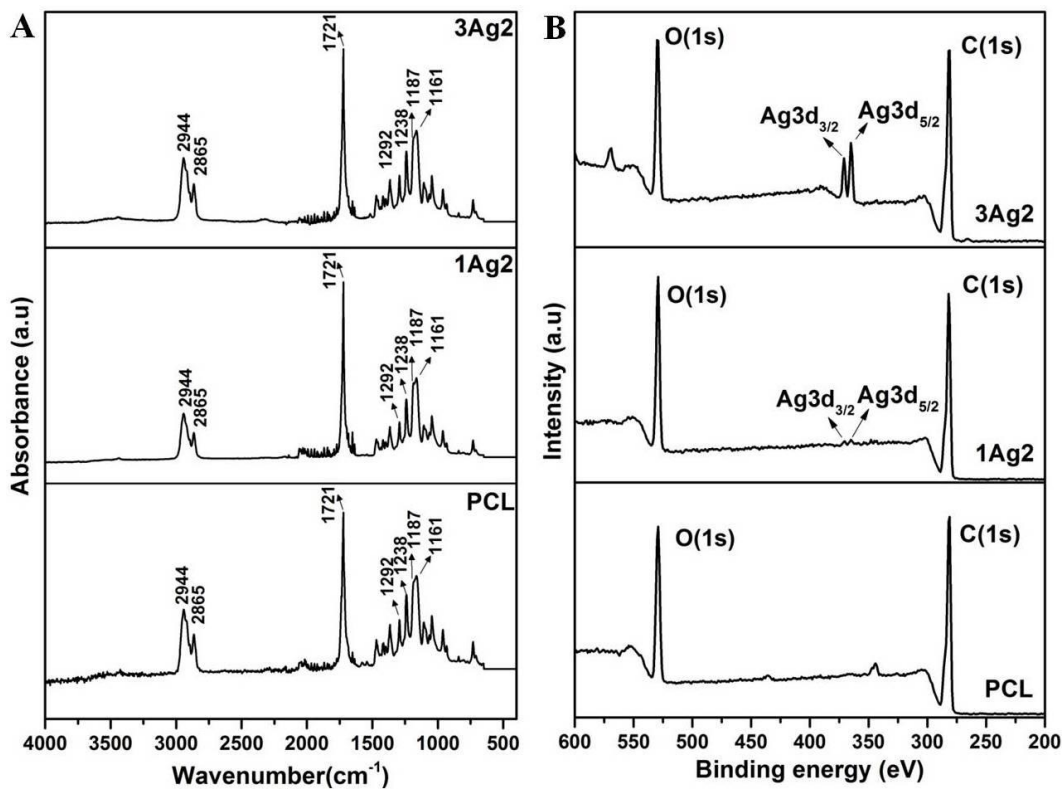
15
 16 **Table 2.** Dimensions of the printed 3D scaffold

	PCL	1Ag2	3Ag2
Pore size (μm)	430.0 ± 29.8	379.6 ± 21.2	431.7 ± 24.6
Filament diameter (μm)	349.1 ± 21.6	406.4 ± 23.3	333.9 ± 12.0

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FTIR analysis was used to identify the functional groups of the 3D printed PCL and PCL/AgNps scaffolds and the interaction between PCL and AgNps. The FTIR spectra are shown in Figure 3(A). The intense peak at 1721 cm^{-1} is assigned to the stretching mode of carbonyl groups of PCL. The peaks at 2865 cm^{-1} and 2944 cm^{-1} correspond to the symmetric and asymmetric stretching of CH_2 groups in PCL polymer backbone. The peaks at 1238 cm^{-1} and 1161 cm^{-1} are due to symmetric and asymmetric stretching of C-O-C groups of PCL. The peaks at 1187 cm^{-1} and 1292 cm^{-1} are related to O-C-O stretching, and C-O and C-C stretching modes respectively³⁴. PCL display polar functional groups $> \text{C}=\text{O}$ and $-\text{C}-\text{O}-\text{C}-$ and their position of the corresponding peaks remains unaffected during the *in situ* reduction of silver ions into silver nanoparticles using DMF³⁵.



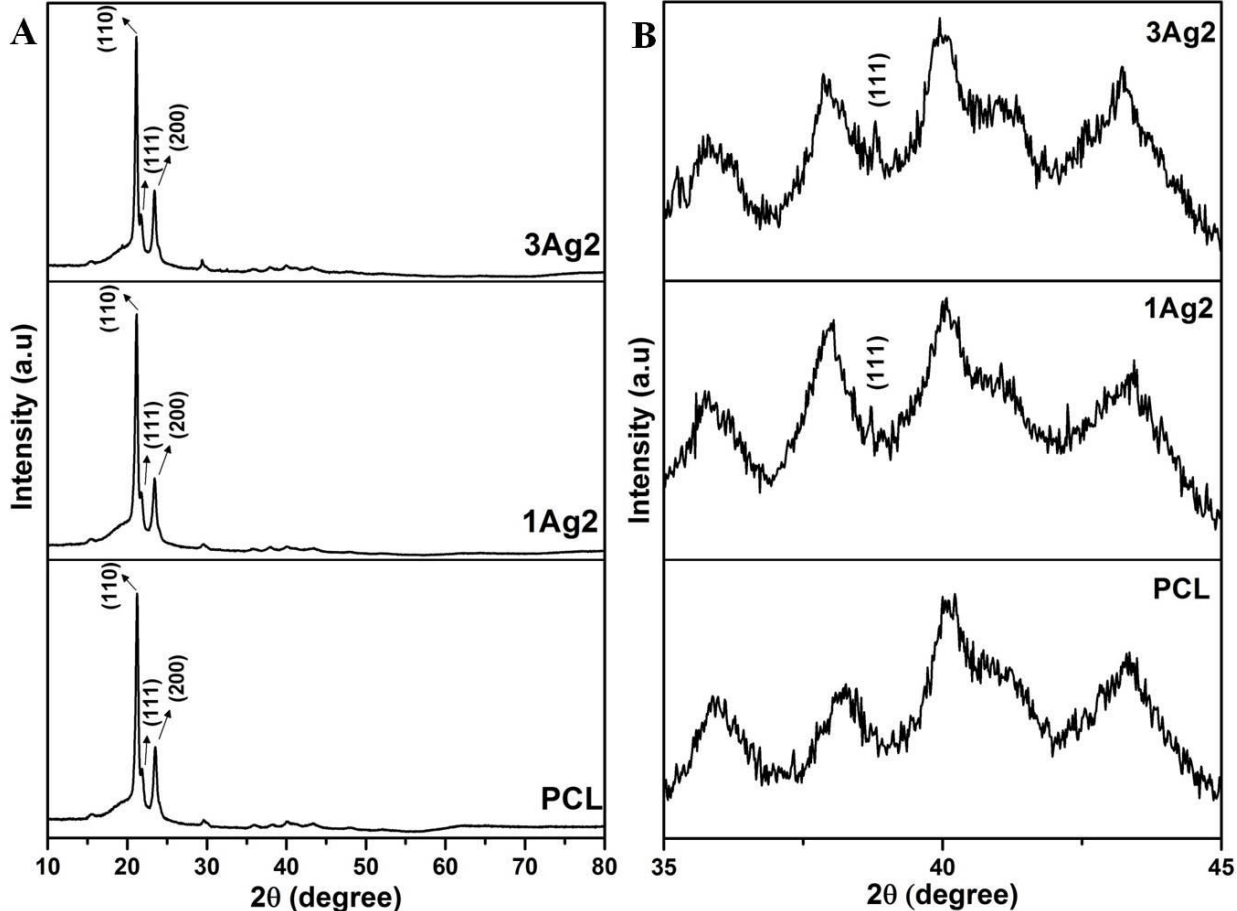
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15 **Figure 3. (A) FTIR and (B) XPS spectra of 3D printed scaffolds**

16

17 X-ray photoelectron spectroscopy (XPS) analysis was used to confirm the formation of silver
18 nanoparticles in the PCL/AgNp composite. The 3D printed scaffolds were scanned in the
19 range of 200 to 600 eV.(figure 3(B)) The XPS spectra showed the peaks of C 1S, and O 1S
20 which correspond to the PCL. The Ag 3d peaks ($3d_{3/2}$, $3d_{5/2}$) are observed in the 1Ag2 and

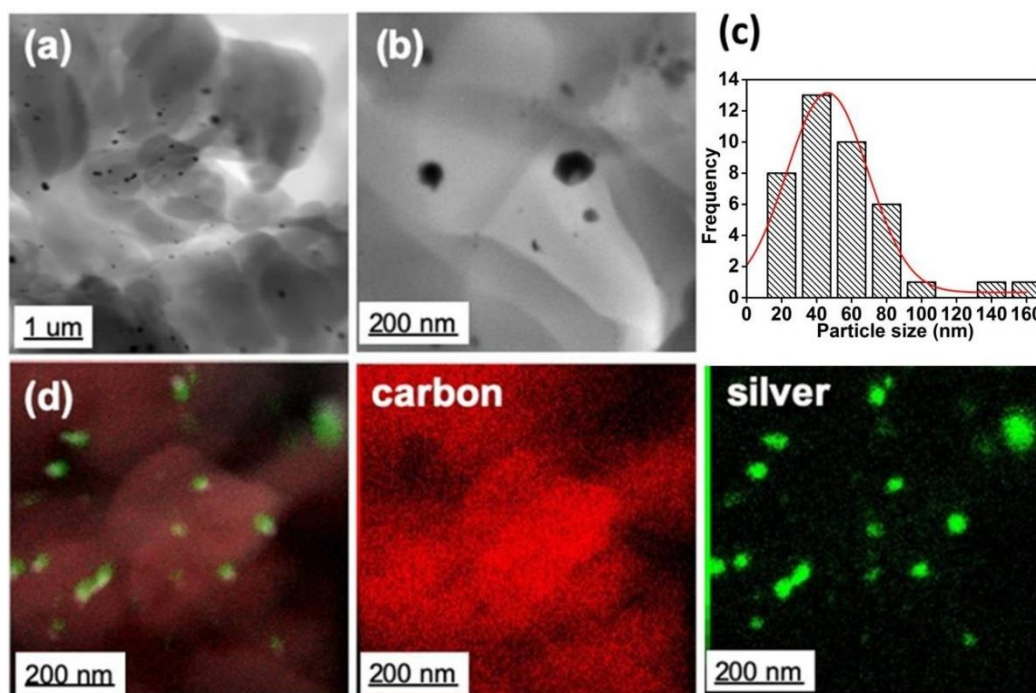
1 3Ag2 samples. They confirm the presence of silver in these scaffolds. The peak separation
2 (energy difference of Ag3d peaks) of Ag 3d_{3/2}, 3d_{5/2} is 6eV which corresponds to the reduced
3 silver. These results show that the reduction of ionic silver into metallic silver is effectively
4 achieved using DMF^{31, 36-38}. Furthermore, silver concentration in 1Ag2 and 3Ag2 was
5 quantified using AAS as 1.34 and 15.22 mg/g of the 3D printed matrix respectively.

6 X-ray diffraction (XRD) was used to determine the crystalline structures of the AgNps/PCL
7 3D printed scaffolds (Figure 4(A)) The XRD diffractogram of the scaffolds PCL, 1Ag2 and
8 3Ag2 are characterized by diffraction peaks at 2 θ = 21.2°, 21.7° and 23.5° which correspond
9 to (110), (111) and (200) crystal planes of PCL respectively³⁹. The diffraction peak observed
10 at 38.8° (Figure 4(B)) in 1Ag2 and 3Ag2 diffractograms corresponds to the (111) plane of
11 Ag. It confirms the presence of silver in 1Ag2 and 3Ag2 scaffolds.



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14 Figure 4. XRD analysis of 3D printed scaffolds, (A) Whole diffractogram and (B)
15 diffractogram of specific regions.
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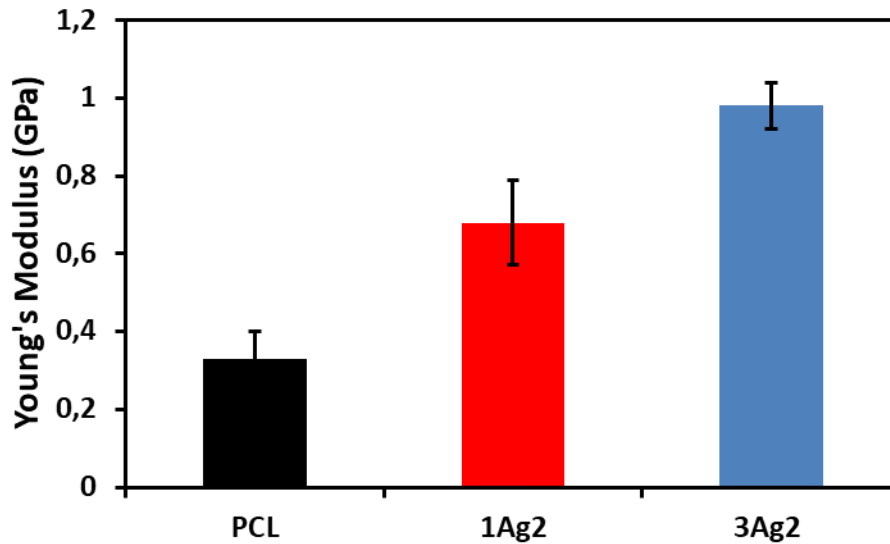
1 *In situ* synthesis and distribution of the silver nanoparticles in the 3D printed PCL
2 matrix was analyzed using transmission electron microscopy (TEM) along with EDX
3 analysis (Figure 5(a-d)). TEM bright field micrographs of 3Ag2 clearly reveal the distribution
4 of silver nanoparticles as spots throughout the PCL matrix (Figure 5(a, b)). The sizes
5 distribution is in the range of 20 to 150 nm (Figure 5c) exemplifying wide dispersity of the
6 synthesized silver nanoparticles. Furthermore, EDX analysis confirms the distribution of
7 silver nanoparticles in the PCL polymer matrix. Therefore, the presence of the silver
8 nanoparticles in the PCL matrix is probably responsible of the antibacterial activity as it will
9 be demonstrated later.



10
11 Figure 5. (a)-(b) TEM images of 3Ag2 sample, (c) Ag particle size distribution and (d) EDX
12 elemental mapping of 3Ag2 scaffolds; Silver (green) and Carbon(red).

13
14 Materials used for implant tissue engineering applications should possess mechanical
15 properties suitable for the targeted tissue. Therefore, the mechanical properties of 3D printed
16 pristine PCL and PCL/Ag were characterized using tensile test analysis (Figure 6). Firstly the
17 Poisson's ratio for PCL was 0.38 and remains constant with the addition of silver
18 nanoparticles. Young's modulus of PCL was found to be 0.35 GPa which is in good
19 agreement with the literature⁴⁰. For PCL/Ag (1Ag2 and 3Ag2), the Young's modulus
20 increases from 0.35 (PCL) to 1 GPa (3Ag2). Generally, the fillers restrict the mobility of
21 polymer matrix and enhance the rigidity of the scaffolds⁴¹⁻⁴². In this study, silver
22 nanoparticles are also acting as fillers which decrease the elastic behavior of PCL and

1 improve its rigidity. Furthermore, increasing the silver concentration enhanced the Young's
2 modulus of the PCL/AgNP (1Ag1 and 3Ag2) composite scaffolds. Here, the values of
3 Young's modulus of the composites are between that of the cancellous bone (0.05-0.1 GPa)
4 and cortical bone (3-30 GPa)⁴³. These results also support the employment of the composite
5 scaffolds for bone tissue engineering application.



6
7 **Figure 6.** Mechanical property, Young's modulus of 3D printed scaffolds.
8

9 **Enzymatic degradation test**

10 *In vitro* enzymatic degradation analysis was used to evaluate stability of the 3D
11 printed PCL/AgNP scaffolds. Lipase was employed because PCL possesses an ester bond
12 prone to hydrolytic degradation by lipase. For this assay, the pre-weighed PCL, 1Ag2 and
13 3Ag2 scaffolds were immersed in the lipase solution and their degradation was recorded
14 (Figure 6). PCL, 1Ag2 and 3Ag2 scaffolds display <10% degradation in 24 hours. The
15 degradation of PCL scaffolds gradually increased to reach 82% degradation after 20 days.
16 Generally, polymer undergoes surface degradation due to the direct contact of enzyme⁴⁴.
17 Scaffolds (PCL, 1Ag2 and 3Ag2) fabricated in this study are interconnected porous scaffolds
18 which display large surface area to interact with enzymes that can facilitate the degradation.
19 Neumann *et al.* 3D printed PCL and PCL/CaCO₃ scaffolds and analyzed the degradation of
20 the scaffolds using 15U/mL lipase. Our material exhibits a degradation profile similar to the
21 degradation profile obtained by Neumann *et al.* in which PCL undergoes 80% degradation in
22 20 days⁴⁵.

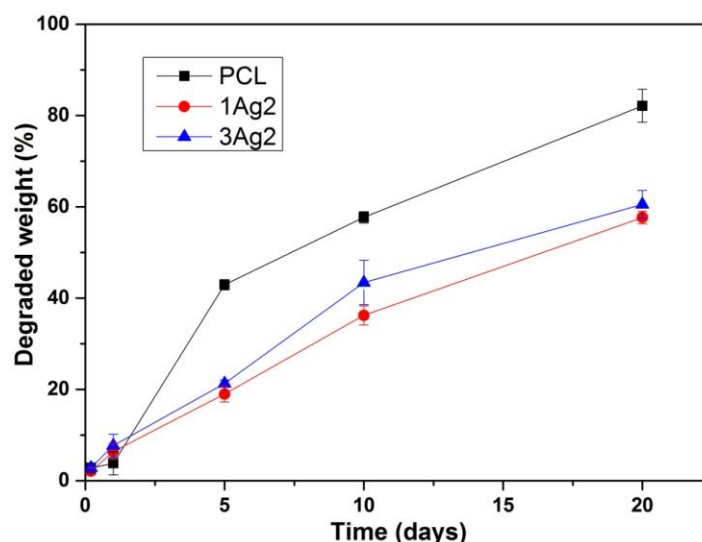
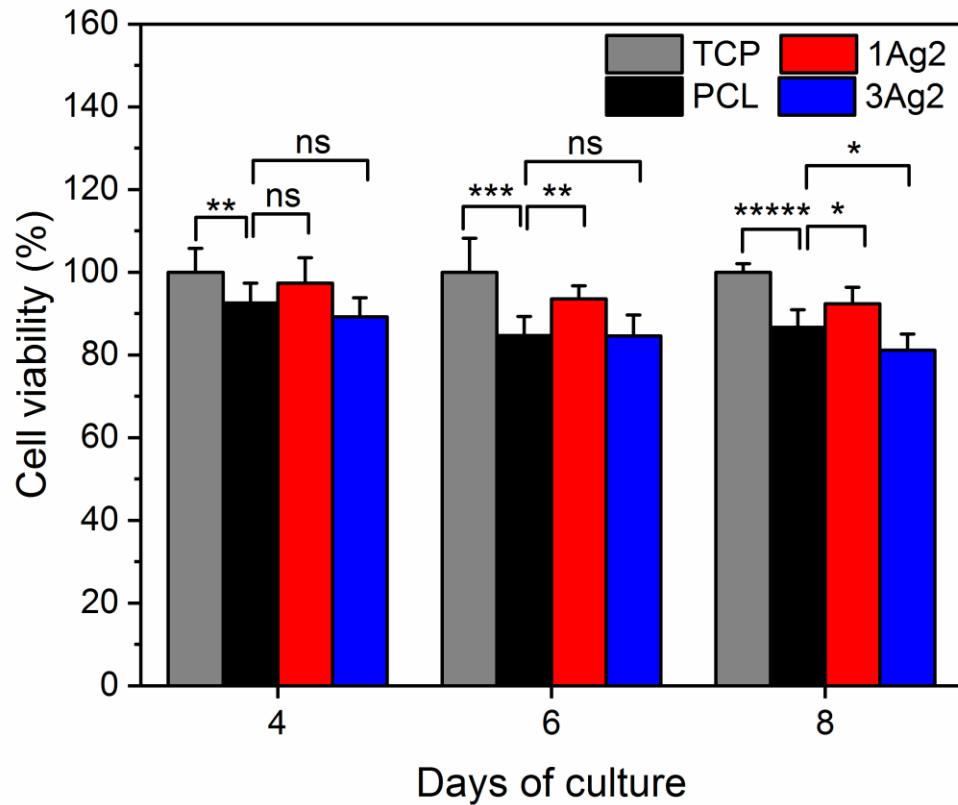


Figure 7. Enzymatic degradation of 3D printed scaffolds

Biocompatibility analysis

Biocompatibility of the PCL, 1Ag2 and 3Ag2 scaffolds was assessed using hFOB cells which are a good *in vitro* model of bone cells (REF?). Cells grown on the tissue culture plate without scaffold served as tissue culture plate control (TCP). Biocompatibility of the 3D printed scaffolds was calculated by comparing cell viability of the 3D printed scaffolds with the TCP control. Cell viability data shown in Figure 8 were presented as mean \pm standard deviation (S.D) and statistical analysis done using Student's t test indicating "ns" not significant and * $p < 0.05$ with sample size, $n=8$.

Afer 8 days of culture, PCL scaffolds did not show any toxicity to the cells which evidences that the solvents employed for the preparation of 3D scaffolds are effectively removed. 1Ag2 allowed a better proliferation of the cells compared than PCL while 3Ag2 display a decrease in cell viability. Cell viability of 3Ag2 observed is likely due to the high concentration of silver loaded in the PCL matrix. However, cell viability was found to be greater than 80 % after 8 days which fits within the acceptable limits as set by ISO10993-5:2009. Despite their toxicity, silver and silver based formulation are utilized for commercial biomedical products such as PolyMem® Silver, Aquacel® Ag, Acticoat™, Urgotul®SSD, Contreet®. Indeed, silver can be eliminated through urinary excretion in humans⁴⁷. Nevertheless, 3D printed PCL/AgNps display low cytotoxicity, suggesting that they could be suitable for tissue engineering application.



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Figure 8. hFOB osteoblastic cell viability after 8 day of culture on of 3D printed scaffolds.

(ns= not significant, * p<0.05, ** p<0.005 and ***** p<0.000005)

6 **Bacterial growth inhibition test**

7 Antibacterial activity of the materials was first characterized by growth inhibition
8 tests. Bacterial growth inhibition test was performed to assess the ability of the material to
9 prevent the implanted site from the formation of bacterial biofilm in presence of nutrients
10 since it could lead to bacterial infection. Figure 9 presents the results obtained for the
11 inhibition tests performed at 24 h.

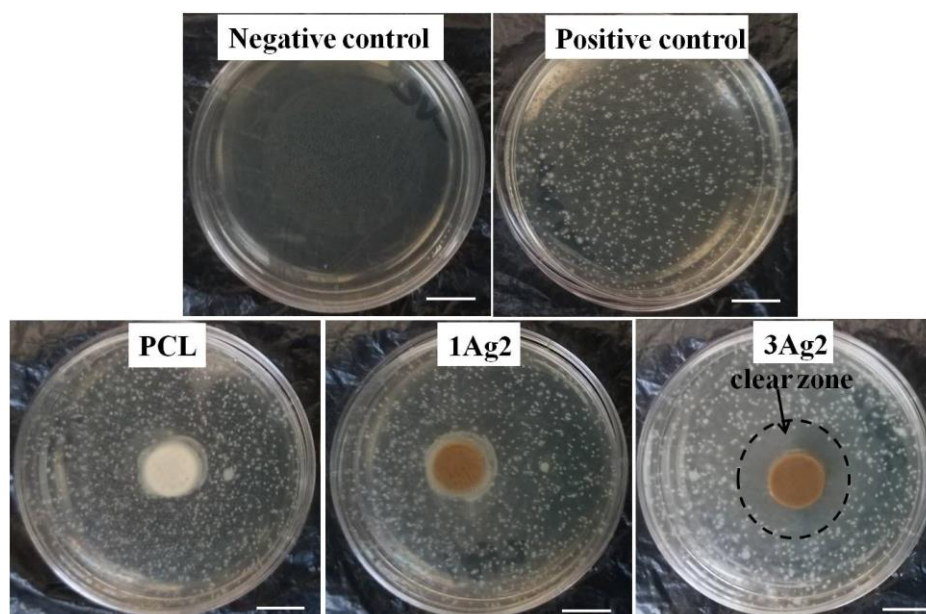


Figure 9. Soft agar plates inoculated with *E. coli* cells and incubated with 3D printed scaffolds in their center for 24 h (Scale bar denotes 1 cm)

Negative control plates (i.e. without bacteria and material) show clear nutritive soft agar plates while positive control plates (i.e., with bacteria and without material) show a uniform distribution of bacterial colonies within the soft agar (Fig. 9). With 3Ag2 material, a clear inhibition zone of mean diameter 20.4 ± 1.7 mm is observed whereas no clear zone is observed for PCL and 1Ag2 (Fig. 9) for which biofilms are evidenced on the material surfaces. The presence of an inhibition zone around the 3Ag2 material evidences an antibacterial activity based on the diffusion of biocidal agents through the soft agar. These biocidal agents might be silver (most likely Ag⁰ rather than Ag⁺ according to XPS analysis) and/or ROS. This antibacterial activity does not exclude a bactericidal surface action by the AgNP present on the material, but the soft agar test does not allow to conclude on that.

Bactericidal activity

Bactericidal tests were done to assess the ability of the material to remove bacteria that has already grown on the material implantation zone. Bactericidal activity was assessed by liquid tests in which the material (90 ± 10 mg) was immersed in a bacterial suspension (10 mL) at about 10^2 CFU/mL. Table 3 shows the bacterial concentrations (CFU/mL) and the associated log-removal values, where the log-removal is defined as the logarithm (log) ratio of the bacterial concentration C measured after respective contact time relative to the initial bacterial concentration C_0 . A log-removal value of $-\log(C_0)$ was attributed to the particular

1 case of total removal of the cultivable bacteria. A log-removal value higher than 1 is related
 2 to a significant bactericidal activity, i.e., to a reduction in bacterial concentration higher than
 3 90 %. Table 3 reports also the total silver concentration measured by AAS in the liquid after
 4 24 h contact.

5

6 **Table 3.** Log-removals and concentrations of released silver obtained in bactericidal tests
 7 against an *E. coli* suspension; the initial bacterial concentration C_0 was 175 ± 50 CFU/mL

Test	After 6 h contact		After 24 h contact		
	Bacterial concentration (CFU/mL)	Log removal (-)	Bacterial concentration (CFU/mL)	Log removal (-)	Total silver concentration (mg/L)
C_{Positive control}	175 ± 50	0	175 ± 50	0	-
C_{PCL}	115 ± 5	0.2	135 ± 0	0.1	-
C_{1Ag2}	145 ± 40	0.1	10 ± 10	1.2	0.06
C_{3Ag2}	5 ± 5	1.5	0 ± 0	2.2	0.44

8

9

10

11 PCL material shows a low log-removal value of 0.2 and 0.1 for 6 h and 24 h contact
 12 time respectively (Table 3). As PCL was not reported to have any antibacterial activity, the
 13 log-removal observed is probably due to bacteria adsorption on the PCL. It is thus likely that
 14 such an adsorption also occurred for the silver materials. After 6 h only, 3Ag2 material shows
 15 a significant decrease of the bacterial concentration corresponding to a log-removal value of
 16 1.5 (Table 3), which is close to the maximal log-removal value reachable (i.e. $-\log C_0 = 2.2$).
 17 Hereafter 24 h, 1Ag2 depicted a marked decrease of bacterial concentration leading to a log-
 18 removal value of 1.2 and 3Ag2 material managed to inactivate all the bacteria initially
 19 present. This result clearly evidences the bactericidal activity of the 1Ag2 and 3Ag2 materials
 20 due to the presence of AgNPs in the material.

21 AAS measurements reveal that small silver concentrations were released in the
 22 suspension after 24 h (Table 3), which corresponds to silver mass lost by the material of
 23 6.0×10^{-4} mg for 1Ag2 and 4.4×10^{-3} for 3Ag2 respectively. Since the initial silver content of
 24 the materials were 0.13 % w/w for 1Ag2 and 1.5 % w/w for 3Ag2, and the samples weight
 25 were 90 ± 10 mg, it means that the mass of silver incorporated in PCL were 0.12 mg for

1 1Ag2 and 1.35 mg for 3Ag2 respectively. After 24 h of reaction, 1Ag2 consequently lost 0.05
2 % of its silver whereas 3Ag2 lost 0.3 %. This result demonstrates the good chemical stability
3 of both PCL/AgNps materials in presence of bacteria. In the case of 1Ag2, a bactericidal
4 action of the surfaces is mainly evidenced because the silver released in the bacterial
5 suspension is very small (< 0.1 mg/L). For 3Ag2, a coupled action of both the AgNps surface
6 and the silver desorbed can be suspected. The need for a longer contact time (24 h vs 6 h) to
7 observe a bactericidal activity of 1Ag2 might consequently be due to a lower content of silver
8 nanoparticles on the material surface contacting the bacteria. These results are consistent with
9 the fact that the antibacterial activity depends on the quantity of silver nanoparticles which is
10 acting as the antibacterial agent⁴⁸.

11

12 **Conclusion**

13 PCL/AgNps polymer composite was achieved through *in situ* reduction of silver ions
14 using DMF as a reducing agent as well as solvent. Synthesized PCL/AgNps composites were
15 extruded into self-standing filaments further utilized for the fabrication of customized 3D
16 porous structures using 3D printing technique. XRD, XPS, TEM and EDAX analysis
17 confirmed the presence of silver nanoparticles within the 3D printed matrix. SEM analysis of
18 the scaffolds shows that the 3D printed scaffolds displayed interconnected porous structures
19 wrinkly surface on PCL and uniform smooth surface on 1Ag2 and 3Ag2 scaffolds. Here the
20 presence of silver nanoparticles in the PCL scaffold improved its stiffness (Young's modulus)
21 and enzymatic stability. Soft agar test showed that 3Ag2 scaffold exhibited an inhibition zone
22 where *E. coli* growth is prevented around the scaffold due presumably to ROS and silver
23 nanoparticles diffusion from PCL scaffold to soft agar. Besides, a biocidal surface action was
24 evidenced for both 1Ag2 and 3Ag2. Furthermore, we show that the 3D printed scaffolds
25 exhibit cytocompatibility. Altogether, our study shows that the scaffolds developed could be
26 utilized for the fabrication of antimicrobial scaffolds for bone tissue engineering application.

27

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