EFFECT OF POLYLACTIDE MODIFICATION WITH β-TCP AND LECITHIN ON THE PROPERTIES OF THE MATERIAL AS A SUBSTRATE FOR OSTEOBLASTS

Radosław Olkowski[1,3,4], Agata Stefanek[2], Piotr Kaszczewski[3], Tomasz Ciach[2], Małgorzata Lewandowska-Szumieł[3,4], Ilona Kalaszczyńska[3,4]*

[1] Department of Pathology,
Center for Biostructure Research,
Medical University of Warsaw,
ul. Chałubińskiego 5, 02-004 Warsaw, Poland
[2] Biomedical Engineering Laboratory,
Faculty of Chemical and Process Engineering,
Warsaw University of Technology,
ul. Waryńskiego 1, 00-645 Warsaw, Poland
[3] Department of Histology and Embryology,
Center for Biostructure Research,
Medical University of Warsaw,
ul. Chałubińskiego 5, 02-004 Warsaw, Poland
[4] Centre for Preclinical Research and Technology,
ul. Banacha 1b, 02-097 Warsaw, Poland
*ikalaszczynska@wum.edu.pl

Abstract

Polylactide (PLLA) containing β-TCP is biodegradable composite and an attractive biomaterial for bone tissue engineering, however, hydrophobicity of PLLA based composites is major limitation for their use as scaffolds for cell culture. In our study lecithin was used to improve hydrophilicity and cytocompatibility of PLLA/ β-TCP composite. Thin films of PLLA, PLLA/ β-TCP and PLLA/β-TCP/lecithin were manufactured by solvent-casting technique. Comparative analysis of all types of films was performed. Addition of β-TCP did not change hydrophilicity of PLLA. The hydrophilicity of PLLA/β-TCP/lecithin increased in comparison to PLLA and PLLA/β-TCP. Degradation of PLLA/β-TCP composite surpassed the degradation of PLLA while addition of lecithin diminished the degradation of composite. The cytocompatibility of composites were studied in 7 day long in vitro assay. Human bone derived cells were seeded on all tested surfaces. Cell viability was estimated by Live/Dead fluorescent staining and Alamar Blue test. Surprisingly, although lecithin addition improved hydrophilicity of the PLLA-based composite, adhesion and proliferation of human bone derived cells were markedly hampered on PLLA/β-TCP/lecithin in comparison to PLLA and PLLA/β-TCP. Despite positive effect we found of lecithin addition on hydrophilicity and stability of PLLA-based composite, its effect on cell attachment and proliferation is negative. Hence, incorporation of lecithin did not improve properties of PLLA/β-TCP/lecithin composite intended for bone tissue regeneration.

***Keywords**: polylactide, lecithin, osteoblasts, scaffold, bone tissue engineering*

[Engineering of Biomaterials 131 (2015) 8-11]

Introduction

Current strategies of tissue engineering are focused on the combination of cells with supportive scaffolds. The biomaterials used to fabricate the *scaffolds need* to be compatible with the cells used for tissue regeneration. Polylactide (PLLA) is a polymer commonly used for scaffold manufacturing for bone tissue regeneration. The main advantage of PLLA is its resorbability. However, significant hydrophobic properties of this material are a cause of poor cell adhesion and its main limitation in bone tissue engineering applications [1]. Moreover, PLLA can elicit an inflammatory response from the host tissue due to its acidic products of degradation [2,3]. Thus, modifications of PLLA by other components admixing or surface modification is desired to improve its properties as a biomaterial for bone regeneration. One of the known and well defined bioactive and osteoconductive ceramic which improve the scaffold properties is β-tricalcium phosphate (β-TCP). Its addition to PLLA allows stabilization of degradation and improvement of biocompatibility in tissue engineering applications [4-6]. Although the PLLA composite with β-TCP is well established and presents higher cytocompatibility *in vitro* than pure PLLA, it remains hydrophobic and unfavorable for cell adhesion and proliferation. Addition of lecithin – natural amphiphilic phospholipid - was shown to improve hydrophilicity and cytocompatibility of PLLA [7]. Therefore, we hypothesized that addition of lecithin to PLLA/β-TCP composite will improve its properties important for application in bone tissue engineering. The physical properties and *in vitro* degradation of the PLLA, PLLA/β-TCP and PLLA/β-TCP/lecithin films were investigated. Further, to test the biocompatibility of the composites, human bone derived cell (hBDC) culture *in vitro* was performed.

Materials and Methods

Preparation of PLLA films

Polylactide (PLLA) of medical purity (Purasorb PL 24, Purac) was dissolved in the 1:1 (v/v) mixture of chloroform (Carlo Erba) and dichloromethane (Chempur) to obtain 30% (w/v) solution. The PLLA solution was used directly to form films and also to prepare other composite materials: PLLA/β-TCP and PLLA/β-TCP/lecithin. The PLLA/β-TCP suspension was prepared by suspending 3 g of β-tricalcium phosphate (Sigma Aldrich) in 100 ml of 30% (w/v) PLLA solution. The PLLA/β-TCP/lecithin solution was prepared by suspending 3 g of β-tricalcium phosphate (Sigma Aldrich) and 1.5 g of lecithin (Serva) in 100 ml of 30% (w/v) PLLA solution. All PLLA suspensions were mixed vigorously for 24 h before use. The PLLA, PLLA/β-TCP, PLLA/β-TCP/lecithin films were formed by pouring the suspension onto a clean glass and forming a layer of controlled thickness (50 μm) using the Elcometer 3700. Films were dried at 37°C until total solvent evaporation and peeled-away. Circles of the diameter 6 mm were cut from the obtained films and used for further experiments and cell culture.

Contact angle study

The contact angle was measured for all three types of obtained PLLA films: PLLA, PLLA/β-TCP, PLLA/β-TCP/lecithin by the use of goniometer (CAM 200, KSV) and the Attension Theta Software (ver. 4.1.0., Biolin, Scientific).

Materials hydrolysis measurement

The composite hydrolysis time was measured for all three types of obtained materials. The 4 circles from each type of PLLA-based films were prepared according to the described protocol. Each circle was incubated in the 50 ml of PBS solution (pH = 7.4) with addition of sodium azide (0.1% w/v) at 37°C in closed container for 4 weeks. The PBS solution was changed every week. After 4 weeks circles were dried until constant mass at 37°C and weighted.

Cell culture

Cytocompatibility of materials was tested *in vitro* on human bone-derived cells (hBDC) isolated from pieces of bone explanted postsurgery. HBDC isolation was performed according to the protocol described previously [8] and was approved by the Local Ethics Committee of the Medical University of Warsaw. Briefly, pieces of bone were cleaned of the connective tissue, cut into fragments of diameter 1-2 mm, rinsed with PBS (Life Technologies) and incubated overnight on magnetic stirrer in medium containing collagenase (Sigma-Aldrich) at 37°C. Bone fragments were then rinsed in PBS, moved into the culture bottles and incubated in Dulbecco's Modified Eagle Medium (DMEM) with 10% addition of Foetal Bovine Serum, 1% L-glutamine, 1% Antibiotic-Antimycotic (all media from Life Technologies) and ascorbic acid (30 µg/ml; Sigma-Aldrich). HBDC were cultured in vitro up to the state of subconfluence, i.e. until cells covered 70-80% of available area in culture bottles. Prior to the experiments cell culture was rinsed with PBS. HBDC were then consecutively incubated in collagenase solution for 20 min., rinsed with PBS and detached from the support with trypsin (Life Technologies).

Samples of biomaterials were placed into wells of 96-well plate and seeded with hBDC. Density of cell seeding was 18 000 cells per cm^2 for Live/Dead staining and 36 000 cells per cm^2 for Alamar Blue assay. *In vitro* culture was continued in DMEM-based medium supplemented with ascorbic acid described above.

Live/Dead staining

HBDC cultured on biomaterial samples for 24 hours were visualized by fluorescent staining with Live/Dead kit (Life Technologies). Living cells converted calcein acetoxymethyl ester into calcein producing green fluorescence. Membrane-impermeant ethidum homodimer-1 labels nucleic acids of membrane-compromised dead cells with red fluorescence. Samples seeded with stained hBDC were observed in fluorescent microscope (Nikon, Japan).

Viability assay

HBDC viability was analyzed by Alamar Blue assay (Life Technologies) [9]. Metabolic activity of living cells is proportional to their redox potential, measured as cell ability to reduce blue, non-fluorescent resazurin to red, fluorescent resorufin. Fluorescence of reaction products was quantified in the ELISA reader (FLUOstar OPTIMA, BMG LABTECH, Germany).

Results and discussion

Contact angle study

The water contact angles of the prepared PLLA films were measured to evaluate the influence of the lecithin addition on the PLLA surface wettability. The results of the contact angle measurements of the three types of materials produced are presented in FIG. 1. The contact angles of the PLLA and for PLLA/β-TCP samples are similar (77° and 79° respectively), thus the addition of the β-TCP has no influence on the surface wettability. As it was expected the value of the PLLA/β-TCP/lecithin surface contact angle is significantly lower (53°) than the contact angle for the PLLA and PLLA/β-TCP surfaces.

Materials hydrolysis

The weight loss of the materials during 4 weeks of incubation in PBS solution is presented in TABLE 1. As it is shown, weight loss of the PLLA/β-TCP composite was significantly higher than of pure PLLA ($p>0.05$). Interestingly, acceleration of composite degradation was evened out by lecithin addition ($p>0.05$). Obtained results were unexpected since in majority of reports, addition of ceramic, e.g. aragonite/vaterite [10], β-TCP [11] to PLLA is shown to slow down the rate of degradation of the polylactide.

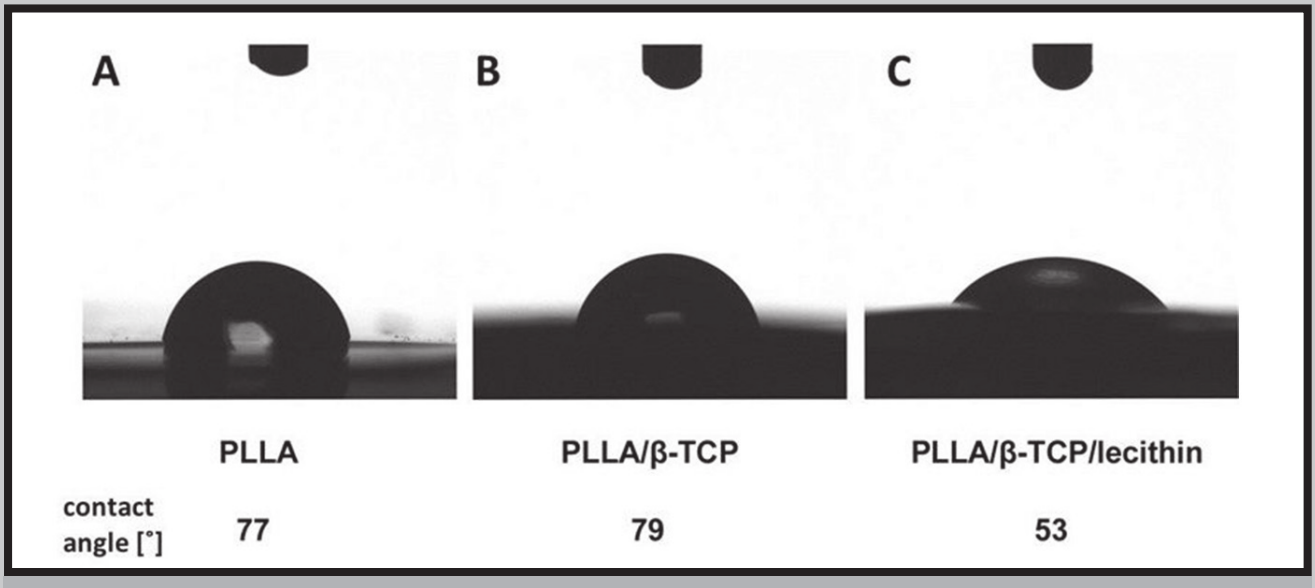

FIG. 1. Contact angle measurements of water droplets on PLLA (A), PLLA/β-TCP (B) and PLLA/β-TCP/lecithin (C) surfaces.

However, this opinion is not univocal since other authors showed that addition of β-TCP to PLLA increases rate of hydrolysis and the process depends on β-TCP concentration [12]. Degradation of polylactide and its composites depends on various factors such as configurational structure, copolymer ratio, crystallinity, molecular weight, morphology, porosity (reviewed in [13]). Therefore, heterogeneity of investigated materials, applied methodology and methods of degradation analysis may explain discrepancies between obtained results (TABLE 1) and reports in literature.

TABLE 1. Degradation rate of the PLLA materials expressed as mean (± standard deviation) weight loss after 4 weeks of incubation in PBS solution, *p<0.05.

Sample	Weight loss [%]
PLLA	6.3 ±0.9
PLLA/β-TCP	9.1 ±0.6*
PLLA/β-TCP/lecithin	7.2 ±2.3*

Viability of hBDC seeded on biomaterial discs

The addition of the 1.5% lecithin improves the hydrophilicity of material surface. Therefore we expected that biocompatibility of the PLLA/β-TCP/lecithin composite will increase as well. Live/Dead staining showed numerous, well spread, alive hBDC on control culture surface as well as on PLLA and PLLA/β-TCP films (FIGs. 2A, B and C). PLLA/β-TCP/lecithin films turned out to be the least cytocompatible – alive cells were less numerous (FIG. 2D). Moreover, presence of spherical and propidium iodide positive hBDC indicated increased cell death.

Quantitative Alamar Blue test confirmed the microscopic observations. Results from day 1 suggest that adhesion of cells to PLLA was less efficient than to control polystyrene surface ($p<0.05$, FIG. 3). In this study, addition of β-TCP improved adherence of hBDC which was not significantly different from that of the control. However, blending the lecithin to the composite reversed positive effect of β-TCP resulting in less than 50% of seeded cell attached to the surface in comparison to control ($p<0.001$). HBDC cultured on PLLA and PLLA/β-TCP films showed similar growth dynamic with significant increase in cell number between day 1 and 7, reaching cell numbers higher than in the control. In contrast, the proliferation of cells, on PLLA/β-TCP/lecithin was significantly hampered and did not change from day 1 of the experiment (FIG. 3).

Decrease in adhesion and proliferation of hBDC on PLLA/β-TCP/lecithin surface were surprising, particularly in view of obtained results showing that addition of lecithin influences increase in hydrophilicity of composite. Xu et al. reported that PLLA containing 5% lecithin were more favorable for mesenchymal stem cells proliferation than pure PLLA or PLLA containing 10% or 15% lecithin [14]. However, searching for explanation of unexpected results we consider influence of external factors, such as light and temperature, on the stability of lecithin. Further, lecithin - unsaturated fatty acid, may undergo oxidation under atmospheric air to form lipid hydroperoxides, resulting in the impairment of its bioactivity and toxicity toward cells [15]. Other possible explanation of low adhesion and impaired proliferation of hBDC is the formation of harmful chemical or physical complexes (microparticles, micelles) of lecithin and calcium phosphate, since lecithin contains phosphoric acid group able to interact with calcium cations. Further, lecithin addition may change mechanical properties of the material [7].

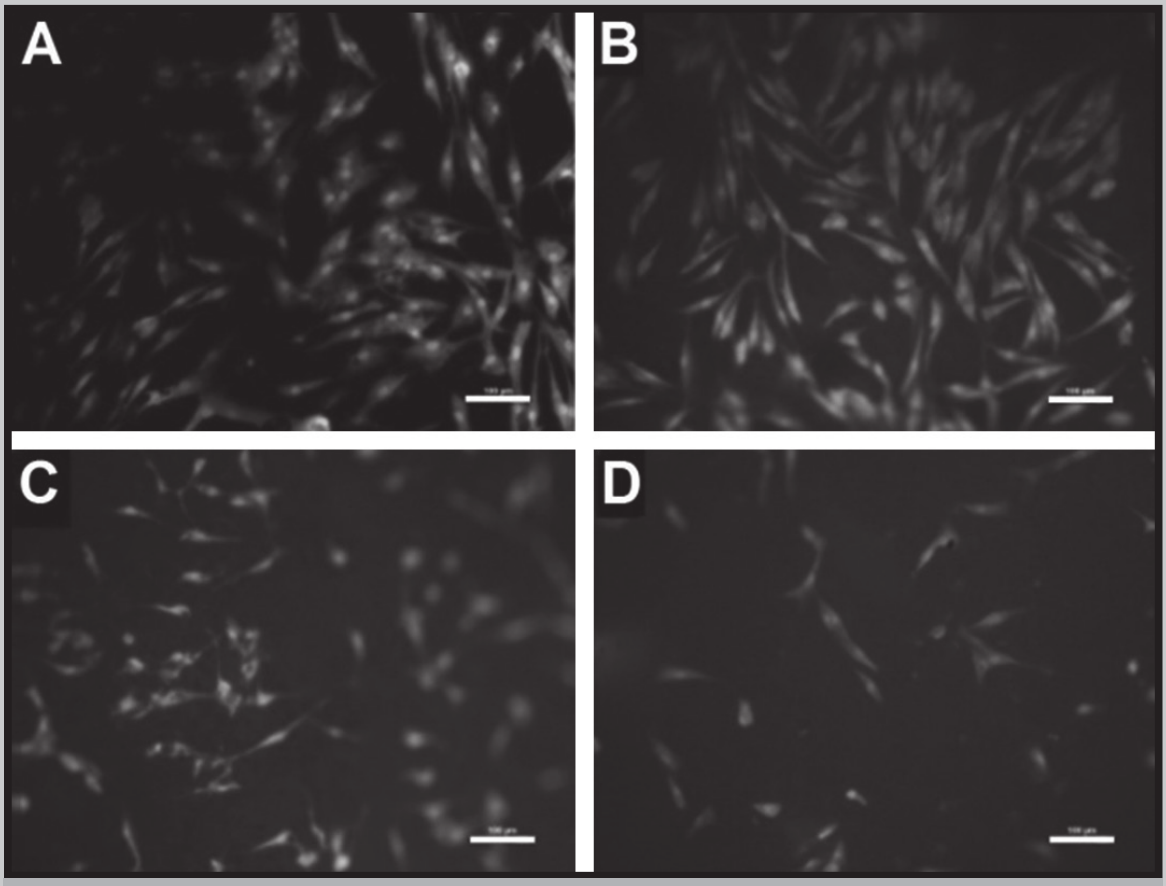

FIG. 2. Analysis of viability of hBDC seeded on culture plastic (A), PLLA (B), PLLA/β-TCP (C) and PLLA/β-TCP/lecithin (D) surfaces. Pictures taken 24 h after seeding.

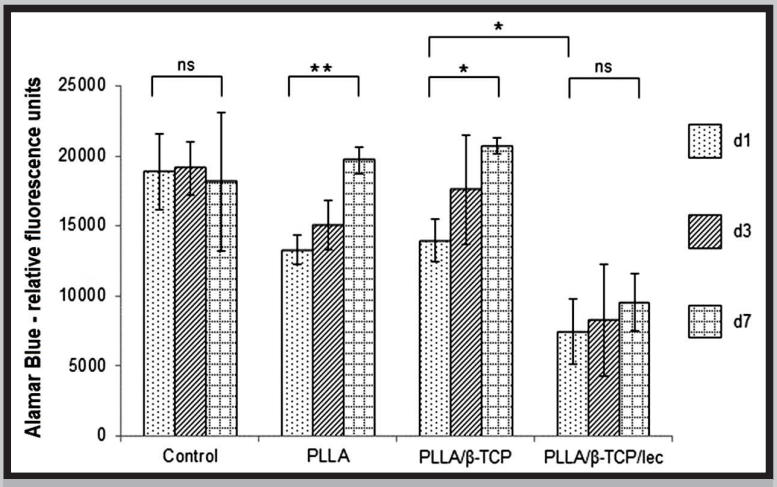

FIG. 3. Viability of hBDC measured by Alamar Blue assay. Data expressed in relative fluorescence units. Error bars show standard deviation. One-way analysis of variance with Tukey's multiple comparisons test was performed to evaluate differences among groups. ** p<0.01, * p<0.05, ns – not significant, lec – lecithin.

Reduced cell attachment and spreading on softer substrata is a known phenomenon [16]. Therefore, surface with higher lecithin concentration, characterized by lower stiffness, might be more beneficial for cell types derived from tissues of low rigidity, but not osteoblasts.

The main message from our study is that despite positive effect of lecithin on surface hydrophilicity, it can have negative effect on cell attachment and proliferation. Therefore functionalization of composites with lecithin may not always be beneficial. These studies have uncovered new unexpected results which need to be addressed further, such as elucidation of lecithin properties as an additive for improvement of composite hydrophilicity.

Conclusions

In this work, we demonstrate that although lecithin addition improved hydrophilicity of the PLLA-based composite, it does not improve adhesion and proliferation of cells. Such statement is supported by Live/Dead staining and viability/proliferation test.

Acknowledgements

This work was supported by National Centre for Research and Development grant NR13-0008-10/2010. The authors have no conflict of interest.

References

[1] Ma Z.W., Gao C.Y., Juan J., Ji J., Gong Y.H., Shen J.C.: Surface modification of poly-L-lactide by photografting of hydrophilic polymers towards improving its hydrophilicity. J Appl Polym Sci 85 (2002) 2163-2171.
[2] Thomas K.A., Toth J.M., Crawford N.R., Seim H.B., 3rd, Shi L.L., Harris M.B., Turner A.S.: Bioresorbable polylactide interbody implants in an ovine anterior cervical discectomy and fusion model: three-year results. Spine 33 (2008) 734-742.
[3] Bostman O., Pihlajamaki H.: Clinical biocompatibility of biodegradable orthopaedic implants for internal fixation: a review. Biomaterials 21 (2000) 2615-2621.
[4] Aunoble S., Clement D., Frayssinet P., Harmand M.F., Le Huec J.C.: Biological performance of a new beta-TCP/PLLA composite material for applications in spine surgery: in vitro and in vivo studies. J Biomed Mat Res. Part A 78 (2006) 416-422.
[5] Bernstein A., Tecklenburg K., Sudkamp P., Mayr H.O.: Adhesion and proliferation of human osteoblast-like cells on different biodegradable implant materials used for graft fixation in ACL-reconstruction. Arch Orthop Trauma Surg 132 (2012) 1637-1645.
[6] Kikuchi M., Koyama Y., Takakuda K., Miyairi H., Shirahama N., Tanaka J.: In vitro change in mechanical strength of beta-tricalcium phosphate/copolymerized poly-L-lactide composites and their application for guided bone regeneration. J Biomed Mat Res 62 (2002) 265-272.
[7] Zhu N., Cui F.Z., Hu K., Zhu L.: Biomedical modification of poly (L-lactide) by blending with lecithin. J Biomed Mat Res. Part A 82 (2007) 455-461.
[8] Kudelska-Mazur D., Lewandowska-Szumiel M., Mazur M., Komender J.: Osteogenic cell contact with biomaterials influences phenotype expression. Cell and Tissue Banking 6 (2005) 55-64.
[9] Schreer A., Tinson C., Sherry J.P., Schirmer K.: Application of Alamar blue/5-carboxyfluorescein diacetate acetoxymethyl ester as a noninvasive cell viability assay in primary hepatocytes from rainbow trout. Analytical Biochemistry 344 (2005) 76-85.
[10] Liu Y.S., Huang Q.L., Kienzle A., Muller W.E.G., Feng Q.L.: In vitro degradation of porous PLLA/pearl powder composite scaff--olds. Mat Sci Eng C-Mater 38 (2014) 227-234.
[11] Imai Y., Fukuzawa A., Watanabe M.: Effect of blending tricalcium phosphate on hydrolytic degradation of a block polyester containing poly(L-lactic acid) segment. J Biomater Sci Polym Ed 10 (1999) 773-786.
[12] Cao L., Duan P.G., Wang H.R., Li X.L., Yuan F.L., Fan Z.Y., Li S.M., Dong J.: Degradation and osteogenic potential of a novel poly(lactic acid)/nano-sized beta-tricalcium phosphate scaffold. Int J Nanomedicine 7 (2012) 5881-5888.
[13] Engineer C., Parikh J., Raval A.: Review on Hydrolytic Degradation Behavior of Biodegradable Polymers from Controlled Drug Delivery System. Trends Biomater. Artif Organs 25 (2011) 79-85.
[14] Xu Z., Wu Q.: Effect of lecithin content blend with poly(L-lactic acid) on viability and proliferation of mesenchymal stem cells. Mater Sci Eng C 29 (2009) 1593-1598.
[15] Marathe G.K., Harrison K.A., Murphy R.C., Prescott S.M., Zimmerman G.A., McIntyre T.M.: Bioactive phospholipid oxidation products. Free Radic Biol Med 28 (2000) 1762-1770.
[16] Discher D.E., Janmey P., Wang Y.L.: Tissue cells feel and respond to the stiffness of their substrate. Science 310 (2005) 1139-1143.